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PLASMID INVOLVEMENT IN ANTIBIOTIC PRODUCTION AND DEVELOPMENT OF CLONING VECTORS FOR USE IN *STREPTOMYCES CLAVULIGERUS*

BY

© LORINDA S. BARRITT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

Fall, 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled PLASMID INVOLVEMENT IN ANTIBIOTIC PRODUCTION AND DEVELOPMENT OF CLONING VECTORS FOR USE IN *STREPTOMYCES CLAVULIGERUS*

submitted by Lorinda Sue Barritt

in partial fulfilment of the requirements for the degree of Master of Science

(Supervisor)

Date: May 9, 1986

ABSTRACT

Four curing procedures were used in an investigation of plasmid involvement in antibiotic production in *Streptomyces clavuligerus*. With the protoplast formation and regeneration procedure, and growth in novobiocin, isolates were obtained which showed a transient inability to produce antibiotic. This lack of antibiotic production also seemed to be correlated to a lack of aerial mycelia. However, no stable antibiotic non-producing isolates were obtained with any of the curing procedures, and the transient inability to produce antibiotic was related to recovery after the curing procedure.

Several techniques were used to demonstrate plasmid DNA in *S. clavuligerus*. Using an alkaline-SDS isolation procedure, there was no evidence of plasmid DNA. Using a neutral lysis procedure, there was some evidence of plasmid DNA in *S. clavuligerus*, but it was not possible to isolate routinely any plasmid.

Transformation of *S. clavuligerus* with pIJ702 isolated from *Streptomyces lividans* was not directly possible. This is presumed to be due to the presence of restriction endonuclease(s). However, pIJ702 was introduced into restriction-deficient protoplasts of *S. clavuligerus*, and it was possible to isolate pIJ702 from these transformants. This modified plasmid DNA was able to transform *S. clavuligerus* at satisfactory frequences of 10^5 -10^6 transformants/μg DNA.

In one instance, pIJ702 isolated from *S. clavuligerus* had increased in size by 1.4kb. This increase in size was due to a fragment of *S. clavuligerus*

chromosomal DNA being inserted into the plasmid.

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ABBREVIATIONS

BHI	brain heart infusion
ccc	covalently closed circular (DNA)
EDTA	ethylenediaminetetraacetic acid
GYEME	glucose-yeast extract-malt extract
MOPS	3-[N-Morpholino]propanesulfonic acid
oc	open circular (DNA)
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSPE	sodium chloride-sodium phosphate-EDTA
TE	Tris-EDTA buffer
TEA	Tris-EDTA-sodium acetate buffer
Tris	tris (hydroxymethyl) aminomethane
TCS	trypticase soy broth
YEME	yeast extract-malt extract

I. INTRODUCTION

Streptomyces spp. are gram positive filamentous bacteria which belong to the family *Actinomycetales*. Actinomycetes are a valuable source of a wide range of antibiotics, producing almost 3000 antibiotic substances. *Streptomyces* spp. make the largest proportion of these substances (Aharonowitz and Cohen 1981). Of these antibiotics produced, many have been found to be useful in human and veterinary medicine, such as streptomycin, chloramphenicol, novobiocin and the tetracyclines. It was only recently that *Streptomyces* spp. were found to produce beta-lactam antibiotics (Nagarajan *et al.* 1971). Prior to that time, production of beta-lactam antibiotics was a characteristic associated with fungi, most notably *Penicillium* spp. and *Cephalosporium* spp. which produce penicillins and cephalosporins respectively.

Of the beta-lactam antibiotic producing *Streptomyces* spp., one that is of particular interest is *Streptomyces clavuligerus*. This organism produces four beta-lactam antibiotics: cephamycin C and desacetoxycephalosporin C (Aharonowitz and Demain 1978); clavulanic acid (Reading and Cole 1976); and penicillin N (Higgens and Kastner 1971). *S. clavuligerus* also produces two additional antibiotics unrelated to beta-lactams, tunicamycin and holomycin (Kenig and Reading 1979).

The biosynthetic pathway of cephamycin C and cephalosporin C has been elucidated in both fungi and streptomycetes (Baldwin *et al.* 1983; Jensen *et al.* 1985). Several of the enzymes involved have been wholly or partially purified and characterized and it has been recognized that there are similarities between the two systems. In contrast, little has been established about the genetics of beta-lactam antibiotic production. The genetically best characterized *Streptomyces* spp. is *Streptomyces coelicolor* A3(2) which produces methylenomycin A. It has been shown that a plasmid, SCP1, codes for the genes involved in methylenomycin A production (Kirby and Hopwood 1977).

With other antibiotics the involvement of plasmids is not so clear. For example, there is no physical evidence for a plasmid in *Streptomyces griseus*, but it appears that cephamycin production (Parag 1979) by this organism involve genes on a plasmid. In other instances, plasmids have been isolated to which no function has been assigned.

In those instances where a plasmid cannot be detected physically, although genetic or biochemical evidence suggests that a plasmid is present, there are several possible explanations. One is that the plasmid may be large and since many of the isolation techniques used for plasmids are harsh, the plasmid is destroyed during the isolation procedure. Another possibility is that the plasmid may become physically entrapped with the chromosome and be lost during the isolation procedure. If a plasmid is involved in antibiotic production, it can be either by coding for regulatory genes, or by coding for the structural genes for biosynthetic enzymes reponsible for antibiotic production (Hornemann and Hopwood 1981). One approach to determining the function of a plasmid is to subject the organism to manipulations which would promote the loss of the plasmid, and then examine for the loss of, or changes in, production of the antibiotic. The manipulations used are called curing techniques, and a number have been shown to be successful in bacteria. With *Streptomyces* spp., a most useful curing technique is protoplast formation and regeneration (Hopwood 1981). Other methods for curing exist, including growth in the presence of novobiocin, growth at elevated temperatures, and growth in the presence of acridine orange (Carlton and Brown 1981).

Regardless of whether the genetic information for antibiotic production is located on a plasmid or the chromosome, it may be beneficial to isolate the biosynthetic and/or the regulatory genes involved. The ultimate aim of this type of work is to manipulate antibiotic production, both in terms of quantity and in the production of novel antibiotics. If the genes involved in antibiotic production are located on a plasmid, then cloning those genes may be straightforward, since the genes already reside on a potentially useful

cloning vector. If, on the other hand, there is no plasmid involved, it is necessary to devise a method for separating and identifying the genes of interest from the rest of the chromosomal material and transferring them to a suitable cloning vector. One such method for cloning genes employs a library of genomic DNA fragments prepared from the producing organism, and inserted into an appropriate cloning vector. The library is then introduced into an appropriate recipient organism that can be used in screening for the activity of the cloned genes either by direct assay (Jones and Hopwood 1984), or by complementation of a mutation (Hopwood 1967; Ohnuki *et al.* 1985; Piret and Chater 1985). Genes involved in the biosynthesis of clavulanic acid in*S. clavuligerus* have been successfully cloned onto a plasmid vector (Bailey *et al.* 1984), and then introduced into a mutant strain of *S. clavuligerus* deficient in the production of clavulanic acid. It was therefore possible to screen for a return to clavulanic acid production as indication that the neccesary genes had been cloned. This work allows for the study of the organization of genes involved in a single biosynthetic pathway.

An essential requirement for these types of procedures is the availability of suitable cloning vectors. A variety of plasmid cloning vectors have been constructed, primarily for use in *Streptomyces lividans*, but their suitability has been demonstrated for other streptomycetes (Bibb *et al.* 1980; Katz *et al.* 1983). To date the *Streptomyces* plasmid most widely used as a cloning vector is pIJ702. This plasmid carries two selectable markers, thiostrepton resistance and melanin production, with suitable single restriction enzyme cleavage sites. The method for introducing a plasmid into a streptomycete involves formation of protoplasts to allow access of the plasmid DNA to the cell membrane, and then addition of polyethylene glycol, which causes the DNA to be absorbed into the cell. This transformation procedure will only be successful if the plasmid is not subjected to the defences of its new host. If there is a restriction-modification system present in the host, in most cases the plasmid will be

digested, and will not survive.

The objectives of this study are threefold:

1. to determine if there is an extrachromosomal DNA element in *S. clavuligerus* which contains on it either the structural genes for the production of cephamycin C, or genes involved in regulation of the biosynthetic pathway

2. to investigate and if necessary overcome restriction-modification barriers in *S. clavuligerus*

3. to establish an efficient transformation system in *S. clavuligerus* in order to further the possibility of gene cloning in *S. clavuligerus* .

II. LITERATURE REVIEW

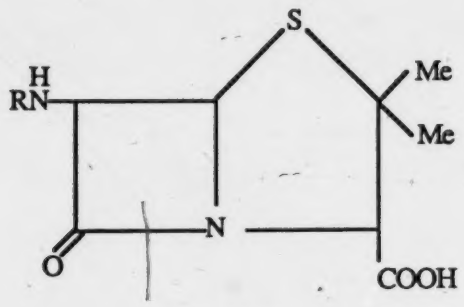
Chemotherapy is the treatment of infectious diseases using chemical substances, with the basic principle being that these chemicals will be selectively more toxic for pathogenic microrganisms than for the cells of the infected host. There are three classes of substances that are used in chemotherapy, and they are: compounds produced by chemical synthesis, natural products derived from plants, and natural products produced by microorganisms. It is within this last group of compounds that most antibiotics are found. At the beginning of the 20th century, there was increasing activity in the fields of chemotherapy and immunology, with the goal of finding therapeutically effective compounds, and this activity continues to the present.

II.1 History of Penicillin

In 1929, Fleming made a report to the British Journal of Experimental Pathology (Fleming 1929) on a substance that inhibited the growth of Gram-positive organisms. The culture filtrate of the mold, *Pencillium notatum*, inhibited the growth of *Staphylococcus*, and was not toxic to animals. Implementation of penicillin as a chemotherapeutic agent was hindered by problems of instability and low productivity (Chain *et al.* 1940; Lovell *et al.* 1932), but by 1943 penicillin was in large scale production, in cooperation with American pharmaceutical companies.

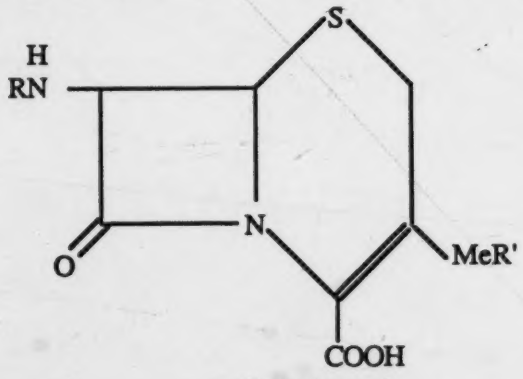
Since Fleming's initial discovery, it has been found that fungi produce many antibiotics, several of which belong to the group of beta-lactams, with penicillin as an example. Several genera of the family *Aspergillaceae* produce beta-lactams. *Penicillium* spp. and *Aspergillus* spp. produce penicillin G, penicillin V, and penicillin O (Hopwood *et al.* 1977). Below is the basic structure for a penicillin-type compound; the R group determines some of the pharmacological properties of the penicillin that results. For example, a phenylacetyl side chain gives penicillin G, an acid sensitive antibiotic with excellent activity against Gram positive bacteria. A phenoxyacetyl side chain gives penicillin V which has similar antibiotic activity to penicillin G, but is acid stable, thus

allowing oral administration. A D-α-aminoadipyl side chain gives penicillin N, a biosynthetic intermediate in the production of cephalosporins and cephamycins (Demain 1983).



Penicillin

Another fungus, *Cephalosporium acremonium*, produces cephalosporin C and penicillins. The production of cephalosporin C by this fungus was the first reported isolation of cephalosporins (Newton and Abraham 1955). These antibiotics have proven to have certain clinical advantages over penicillin due to their broader activity spectrum and greater resistance to degradation by enzymes. Shown here is the basic structure for cephalosporin-type compounds. As with penicillins, different side chains for R and R' give each cephalosporin different properties. The D-α-aminoadipyl side chain for R and $-OCOCH_3$ for R' gives cephalosporin C, while desacetoxycephalosporin C has the



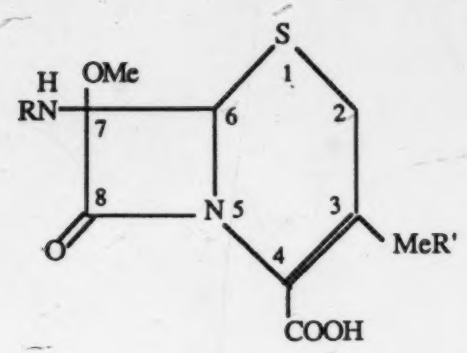
Cephalosporin

same R side chain but R' is simply -H.

II.2 Isolation of Antibiotics from *Streptomyces* spp.

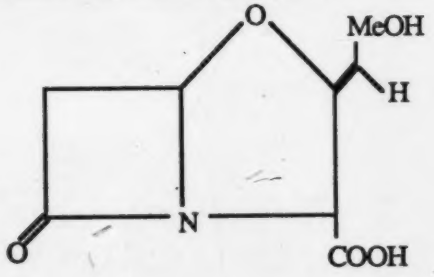
At the same time that Chain and his co-workers were studying penicillin, Waksman was beginning his work of surveying soil microorganisms for antibiotics. The first antibiotic he discovered to be produced by the *Actinomycetales* was actinomycin (Waksman and Woodruff 1940). In subsequent studies, Waksman described several more antibiotics including streptothricin, streptomycin and neomycin. Not all of the substances isolated were of clinical value, but in 1952 Waksman received the Nobel Prize for his discovery of streptomycin. At present, *Streptomyces* spp. are known to produce in excess of 3000 antibiotic substances of which about 50 are produced commercially (Fleck 1979).

Waksman made a large contribution to the study of soil microorganisms by stressing the potential of the *Actinomycetales* as a source of antibiotics, but it was not until 1971 that their potential to produce beta-lactam antibiotics was recognized (Nagarajan *et al.* 1971). Since the discovery that beta-lactams were produced by *Streptomyces* spp., increased attention has gone to these organisms. *Streptomyces* spp. are prokaryotic organisms, with less complexity than the fungi thus they can be used as a model system to study antibiotic production. The classical beta-lactam compounds that they produce are: penicillin N; a cephalosporin type antibiotic, deacetoxycephalosporin C; and cephamycin type antibiotics. The structure for cephamycin-type compounds is shown below. Cephamycins differ from cephalosporins in that they contain an $-OCH_3$ group at C7. Again different R and R' side chains are possible. For cephamycin A, R is a D-α-aminoadipyl side chain, and R' is $-OCOCH(OCH_3)-CH_2-C_6H_6-OSO_3H$. Cephamycin C has the same R side chain as cephamycin A with $-OCONH_2$ for R' (Elander 1975).

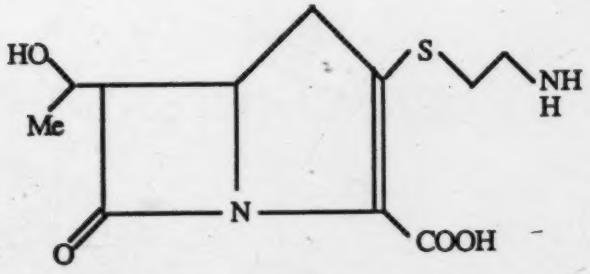


Cephamycin

Recently, *Streptomyces* species have also been discovered to produce a number of novel beta-lactam compounds, including clavulanic acid, and carbapenem compounds such as thienamycin and olivanic acid (Elander 1975). Clavulanic acid is a beta-lactam compound with no antibacterial activity of its own, but it has the ability to bind irreversibly with beta-lactamases, so clavulanic acid behaves as an inhibitor of beta-lactamases. Thienamycin has both antibacterial activity and beta-lactamase inhibitor activity. Below are clavulanic acid and thienamycin:



Clavulanic acid



Thienamycin

Streptomyces clavuligerus was isolated from a South African soil and synthesizes penicillin N, cephamycin C (Higgens and Kastner 1971), and clavulanic acid (Reading and Cole 1976), along with tunicamycin and holomycin (Kenig and Reading 1979).

II.3 Studying Beta-Lactam Production

Some work has been done to understand the biosynthesis of penicillins, cephalosporins and cephamycins. With the successful preparation of cell-free extracts of both *C. acremonium* and *S. clavuligerus* it has been possible to observe the activity of the enzymes involved in antibiotic synthesis (Jensen *et al.* 1985; Kupka *et al.* 1983). In general, the first step in the synthesis is a condensation of L-α-aminoadipic acid with L-cysteine to give a dipeptide. Following this, the carboxyl group of the cysteinyl residue condenses with L-valine. During this reaction L-valine is converted to the D-configuration and the resulting tripeptide is δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). An enzyme, isopenicillin N synthetase then converts ACV through an oxidation reaction, to isopenicillin N. An epimerase enzyme takes isopenicillin N, with the L-α-aminoadipyl side chain, to penicillin N with the D-configuration of the side chain. An "expandase" enzyme enlarges the 5-membered thiazolidine ring to a 6-membered dihydrothiazine ring to form desacetoxycephalosporin C and a "hydroxylase" enzyme converts this to desacetylcephalosporin C. From here the pathway splits, going to cephalosporin C, or cephamycin C.

These studies have shown that there are similarities between the *C. acremonium* and *S. clavuligerus* biosynthetic pathways leading to the production of penicillins and cephalosporins. In particular, the enzyme involved in the conversion of ACV to isopenicillin N in both of these organisms has been shown to have similar molecular weights and similar requirements for O_2, ascorbate and ferrous ion (Jensen *et al.* 1982a; Jensen *et al.* 1982b; Baldwin *et al.* 1983).

Although some of the biochemical aspects of antibiotic production have been investigated, less is known about the genetic aspects. This is due largely to the fact that until recently genetic manipulation at the industrial level was restricted to mutagenesis used to achieve strain improvement. As a result, very few of the industrial antibiotic producing organisms have well characterized genetic systems (Hopwood and Merrick 1977).

In order to take full advantage of the capabilities of antibiotic producing microorganisms, a complete understanding of the genetic aspects of antibiotic production is necessary. *Streptomyces coelicolor* A3(2) is the best genetically characterized streptomycete with a well developed chromosomal map, and has been used as a model system for *Streptomyces* spp. The linkage map has been determined through mixed culture mating of complementary parental strains. A plasmid, SCP1 has been demonstrated to act as a fertility plasmid, promoting the exchange of chromosomal DNA. One hundred loci have been mapped to the chromosome. Arrangement of markers on the chromosomes of *Streptomyces glaucescens*, *Streptomyces rimosus*, *Streptomyces olivaceus*, and *Streptomyces bikiniensis* bear strong similarities to *S. coelicolor* A3(2) (Hopwood *et al.* 1973).

II.4 Involvement of Plasmids in Antibiotic Production

One of the first steps in analyzing genes involved in antibiotic production is to determine their location and then determine what controls the expression of these genes. In this regard, the involvement of plasmids needs to be determined. Hopwood (1978) outlined what is acceptable evidence for a plasmid determined characteristic. There are three lines of evidence for procaryotic organisms. First, a plasmid may be self-transmissible or transmissible along with a second plasmid. Therefore, genes can be determined to be plasmid borne if their frequency of transfer is significantly higher than that observed for chromosomal recombination, a phenomenon sometimes referred

to as infectious transfer. Hopwood cautioned that failure to observe this phenomenon does not preclude plasmid involvment, as some plasmids are non-transmissible. Also, infectious transfer on its own, is not substantial as evidence, as some plasmids promote the transfer of certain regions of the chromosome, resulting in frequencies greater than that for normal chromosomal recombination. A second line of evidence is the correlation of a particular characteristic with the physical presence of a plasmid. Again, negative evidence is inconclusive as some genetically well characterized plasmids are difficult to detect physically, such as the *S. coelicolor* A3(2) plasmid SCP1. The third line of evidence is instability and curing. Phenotypic instability refers to the appearance of stable antibiotic non-producing variants that arise at a frequency higher than that of random gene mutations, and is an indication that the genes are plasmid borne. This can be further substantiated if the instability increases with the use of procedures that promote the loss of the plasmid. Again, a negative curing result on its own is insufficient as evidence against plasmid involvement since plasmids that occur in high copy number are lost infrequently (Hopwood 1978).

Three apparent patterns of genetic control have been observed with antibiotic production. In the first situation plasmids are not involved. All mutants generated, which interrupt the synthesis of an antibiotic, can be mapped to the chromosome. An antibiotic of this nature is actinorhodin, produced by *S. coelicolor* A3(2). The mutations of actinorhodin production also map very close to one another on the chromosome indicating the possibility of the arrangement of antibiotic production genes into operons (Hornemann and Hopwood 1981).

The second apparent pattern has some or all of the structural genes responsible for antibiotic production located on the chromosome, but with plasmids apparently influencing gene expression. This is usually manifested as unstable antibiotic production where the loss of a plasmid, either spontaneous or induced, causes a

significant reduction but not a complete loss of antibiotic production. Some examples are oxytetracycline production in *Streptomyces rimosus*, and chloramphenicol production in *Streptomyces venezuelae* (Hornemann and Hopwood 1981). Holomycin production in *S. clavuligerus* has also been observed to be unstable. Nonproducing isolates occur on solid medium at a spontaneous frequency of 0.5% which can be increased to 3% after curing procedures, and transfer of the holomycin producing phenotype occurs at about 15% (Hopwood 1978). Hopwood (1978) has noted that a holomycin nonproducing isolate could produce holomycin in liquid culture, suggesting that an unstable element regulates the production of holomycin, but that the biosynthetic genes are chromosomal (Hopwood 1978; Kirby 1978).

The third pattern observed is where the genes required for antibiotic synthesis are mapped to a plasmid. An example of this pattern is the production of methylenomycin A by *S. coelicolor* A3(2). The information required for production of this antibiotic is carried on the plasmid SCP1 (Hornemann and Hopwood 1981).

II.5 Plasmid Isolation and Detection

There are a number of techniques available for the detection of plasmid DNA (Chater *et al.* 1982). Plasmids may be isolated from *Streptomyces* spp. by a variety of methods, three of which will be described in some detail; all of the methods involve treatment of the mycelia with lysozyme. The alkaline-sodium dodecyl sulfate (SDS) procedure involves lysing the mycelia after lysozyme treatment and removing most of the chromosomal DNA by treatment with alkali. This procedure is most successful with small plasmids. The neutral lysis procedure is different from the alkaline-SDS in that the pH of the preparation remains neutral, and the plasmid is separated from the chromosomal DNA by precipitation of the chromosomal DNA with salt and polyethylene glycol. The third procedure involves the formation of protoplasts by the lysozyme treatment. Protoplasts lack a cell wall, and may be lysed in a controlled manner for

plasmid isolation (Chater *et al.* 1982).

In a survey of *Streptomyces* spp. Kirby *et al.* used a neutral lysis procedure to isolate plasmid DNA (Kirby *et al.* 1982), and then he attempted to observe the presence of the plasmids by use of caesium chloride-ethidium bromide density gradient centrifugation, agarose gel electrophoresis and electron microscopy. The purpose of Kirby's study was to isolate plasmids which might be useful as cloning vectors. Of the 23 species tested, only 8 showed any sign of plasmid DNA and from this Kirby concluded the presence of plasmids was not ubiquitous. One of the tested species was *S. clavuligerus* ; it was not found to contain a plasmid (Kirby *et al.* 1982). There are reports of a variety of *Streptomyces* spp. which contain plasmid DNA: *S. coelicolor* A3(2) (Bibb *et al.* 1977; Schrempf *et al.* 1975); *Streptomyces kasugaensis* (Okanishi 1977); *S. venezuelae* 3022a (Malek 1977); *Streptomyces fradiae* (Yagisawa *et al.* 1978), and *Streptomyces lividans* (Bibb *et al.* 1980). Similarly, Pernodet and Guerin examined *Streptomyces coelicolor* isolates for plasmid DNA (Pernodet and Guerineau 1981). Plasmid DNA was prepared by a neutral lysis procedure, and isolated by agarose gel electrophoresis. In their examination, the two isolates tested contained a plasmid.

A variety of self-transmissible plasmids have been detected by the formation of what is called a pock (Kobayashi *et al.* 1984). A pock is formed when a plasmid bearing strain is grown with a plasmidless strain. Around the plasmid bearing strain a temporary zone of growth inhibition of the plasmidless strain occurs as the plasmid is being transferred. It was the observation of pocks that eventually led to the isolation of the self-transmissible plasmid, SCP2 of *S. coelicolor* A3(2). The formation of pocks, also termed lethal zygosis, although there is no bacteriocidal effect, has aided in the detection of low frequency interspecific transfer of SCP2. In other instances this observation of pocks suggests that a plasmid may be present even if it cannot be physically isolated (Westpheling 1980).

An interesting family of plasmids has been detected in *S. lividans*. This SLP1 series of plasmids was generated by cleaving total DNA of *S. coelicolor* with a restriction enzyme, ligating the ends of the fragments of DNA and introducing them into *S.lividans* (Bibb *et al.* 1981). Some portion of the circularized DNA fragments persisted in *S. lividans* as plasmids. The plasmids subsequently isolated from *S. lividans* varied in size from 9.38 kilobases (kb) to 12.35 kb with the 9.38 kb sequence being common to all of them.

In a similar situation, a plasmid free culture of *S. lividans* was grown in mixed culture, with a plasmid free culture of *Streptomyces parvulus*. Transfer of chromosomal DNA between strains occurred at a low frequency by conjugation. Some of the transconjugants of *S. lividans* that resulted, were able to form pocks on the original plasmid free *S. lividans* culture. A plasmid was isolated from the *S. lividans* transconjugants and determined to have originated from the chromosome of *S. parvulus* (Hopwood *et al.* 1984). This would suggest that some sequences of DNA may be present as part of the chromosome in certain *Streptomyces* species and as free plasmids in other species.

Another interesting occurance in a few *Streptomyces* spp. is the presence of extrachromasomal DNA which is linear instead of covalently closed and circular in nature. The first example of linear plasmids was demonstrated with a *Streptomyces* spp. that produced lankacidin-type antibiotics. The linear DNA was isolated by agarose gel electrophoresis and then purified by hydroxyapatite column chromatography (Hayakawa *et al.* 1979). Factors that led to the conclusion that this segment of DNA was indeed linear were: the DNA could not be separated from chromosomal DNA on caesium chloride-ethidium bromide density gradient centrifugation; a second distinct band of DNA was observed on ethidium bromide stained agarose gels; electron microscopy showed the DNA segment to be linear. This segment was termed a linear

plasmid, pSLA2, on the basis that cleavage with restriction endonucleases gave rise to distinct DNA fragments whose sum of molecular weights equalled that of the complete pSLA2 and that the host could be cured of the plasmid by treatment with ethidium bromide (Hayakawa *et al.* 1979). Ethidium bromide has been demonstrated to be capable of causing the loss of plasmid DNA (Bouanchaud *et al.* 1969). The ends of this plasmid contain a 614 base pair inverted terminal repeat, and are associated with protein (Hirochika *et al.* 1982; Hirochika *et al.* 1984). The association of the ends of the plasmid with protein plays a key role in the isolation of the plasmid in that it is necessary to remove the protein before removing cell debris and proceeding with the isolation of the DNA. Recently, a similar linear plasmid of unknown function has been detected in *S. clavuligerus* (Keen 1985).

II.6 Curing Procedures

Curing a host of its plasmid involves treating the host with a chemical, or subjecting it to unusual growth conditions in such a manner as to promote the loss of the plasmid. Several methods are available such as growth at temperatures 4° to 5°C above the optimal growth temperature of the organism. The effect of this may by related to changes in the membrane of the cell, and would therefore be effective for curing plasmids associated with membranes (Carlton and Brown 1981), or growth at elevated temperatures can also result in complete or partial deletions (Trevors 1986). Another condition used for plasmid curing is growth in the presence of compounds which at higher concentrations are mutagenic. Examples are ethidium bromide (Bouanchaud *et al.* 1969), novobiocin, acriflavin and acridine orange. Ethidium bromide, acriflavin and acridine orange are intercalating dyes, and will inhibit plasmid replication (Trevors 1986). Novobiocin inhibits supercoiling of plasmids dependent on DNA gyrase (Trevors 1986). One of the most effective curing techniques for *Streptomyces* is the formation and regeneration of protoplasts. There are several possible explanations for

the curing ability of protoplast formation and regeneration: plasmids may be associated with mesosomes and would therefore be lost upon protoplasting, as mesosomes tend to be expelled during the removal of the cell wall; subdivision of the mycelium into protoplasts may conceivably generate protoplasts which do not carry a plasmid; regeneration of protoplasts may give rise to developing hyphae which fail to include a plasmid copy (Hopwood 1981). Once a culture has been cured of its plasmid it is then possible to determine the effect of that loss on antibiotic production. This can be done by assaying for the antibiotic and comparing production in cured versus plasmid bearing strains.

II.7 Protoplast Formation and Regeneration

Protoplast formation and regeneration is an important technique for genetic studies with *Streptomyces* spp. In addition to use as a curing technique, protoplast formation and regeneration is also an essential step in genetic recombination by protoplast fusion and in transformation. Okanishi *et al.* (1974) established a procedure for the formation of protoplasts and their regeneration. The technique involves the suspension of mycelia in isotonic media in the presence of lysozyme, which degrades the cell wall. The protoplasts are regenerated on solid medium which is also isotonic, preventing the protoplasts from bursting (Okanishi *et al.* 1974).

In genetic recombination procedures, protoplasts can be fused in the presence of polyethylene glycol. This fusion significantly increases the frequency of genetic recombination over that seen when intact cells are co-cultivated on solid medium. The use of protoplast fusions also allows for the recombination to occur between more than two genomes since more than two protoplasts can fuse at the same time (Hopwood and Wright 1978).

In transformation procedures, plasmid DNA can be introduced into protoplasts by incubating the protoplasts with the plasmid in the presence of polyethylene glycol (Bibb

et al. 1978). In this case the concentration of polyethylene glycol used is lower than that required to give protoplast fusion, about 20% as opposed to 60% (Hopwood and Wright 1979). The actual manner by which polyethylene glycol stimulates plasmid DNA uptake or protoplast fusion is unknown.

II.8 Cloning in *Streptomyces* spp.

The once unattainable goal of isolation and identification of genes involved in the synthesis of antibiotics has now become realistic with the development of recombinant DNA techniques for use with *Streptomyces* spp. With techniques now available, researchers have begun to examine the structure and control of genes responsible for antibiotic production, and eventually hope to be able to alter the quantity and type of gene products in a controlled fashion. There is an increasing number of reports of successful cloning experiments in the literature. A successful cloning system generally requires the use of a selectable cloning vector and a method of generating suitable fragments of the DNA containing the gene to be cloned. The fragments obtained are inserted into the vector in a location that allows for differentiation of transformants carrying the vector with inserted DNA from transformants carrying the unmodified vector. This general procedure, referred to as shotgun cloning, has been used to clone *Streptomyces* spp.genes into the same as well as other species.

Bibb *et al.* (1980) developed an interspecies gene transfer system by using the plasmid vector, SLP1, and cloning into it the gene for resistance to thiostrepton from *Streptomyces azureus* (Bibb *et al.* 1980). Using a derivative of this plasmid, called pIJ101, other workers have been able to introduce the tyrosinase gene from *Streptomyces antibioticus.* The plasmid pIJ101 is a self-transmissible plasmid which produces the characteristic of lethal zygosis (Kieser *et al.* 1982). The tyrosinase gene is responsible for the production of melanin and gives rise to black colored colonies. The new construct, called pIJ702, was introduced to *S. lividans* where the production of

melanin and thiostrepton resistance was detected (Katz *et al.* 1983). The plasmid pIJ702 has a copy number of 40 - 300 per genome, and lacks the conjugative functions of pIJ101. There is a single Bgl II restriction site which is located within the tyrosinase gene. Insertion of DNA fragments at this site interrupts the tyrosinase gene giving rise to white colonies. This works as a selectable marker when cloning. The host range of this plasmid has been examined, and it appears to be maintained in a wide range of *Streptomyces* spp. (Katz *et al.* 1983).

There has been a number of cloning studies which have used pIJ702 as the cloning vector. The gene for O-methyl transferase, involved in the production of undecylprodigiosin, was isolated from the chromosomal DNA of *S. coelicolor* by inserting chromosomal fragments into the Bgl II restriction enzyme site of pIJ702. The hybrid plasmid was introduced into a mutant of *S. coelicolor*, blocked for the synthesis of undecylprodigiosin, and in a single isolate, production was restored (Hopwood *et al.* 1983). In a second example, the regulation of candicidin biosynthesis in *S. griseus* was studied by using pIJ702 to reintroduce the cloned genes of the pathway, at a high copy number, to *S. griseus* (Hopwood *et al.* 1983).

In the first two examples it was possible to detect transformants containing plasmids with an insertion as the production of tyrosinase was lost resulting in white colonies. Bailey *et al.* (1984) have been able to isolate genes involved in the biosynthesis of clavulanic acid using pIJ702 as a cloning vector. The difficulty they encountered was that the melanin phenotype was not expressed in *S. clavuligerus*, and so they were unable to distinguish transformants with unmodified plasmid from those with plasmids bearing inserts. However, it was still possible to transform *S. lividans* and determine the frequency of insertion; they determined the frequency of insertion to be approximately 95%.

II.9 Restriction and Modification

Many organisms have a means by which they protect themselves against foreign DNA, for example the DNA of a virus. The organism's own DNA is modified in a specific manner so that it is recognized as self. Along with the modification there is also a specific enzyme that recognizes the absence of the modification, and cleaves unmodified DNA; this enzyme is a restriction enzyme. It is very common for soil organisms to have both types of enzymes, resulting in what is known as a restriction/modification system. The presence of restriction enzymes gives rise to barriers to interspecific gene cloning, and limits the host range of plasmids and actinophage which can be used as cloning vectors (Cox and Baltz 1984). It is possible to determine if a restriction enzyme is present by observing the failure of actinophage to produce plaques (Cox and Baltz 1984), or the inability of a plasmid to transform (Hunter, personal communication). A *Streptomyces* spp. may contain more than one restriction enzyme (Sanchez *et al.* 1985), with as many as five (Hunter, personal communication).

In order to transform an organism with unmodified DNA, such as a plasmid from another organism, it becomes necessary to overcome the restriction/modification system of the organism. There are two possible ways in which this can be done. One is to treat the cells so that the restriction enzyme is not produced, but the modification enzyme remains unchanged. When foreign plasmid DNA is introduced it will not undergo restriction and will become modified. This modified plasmid DNA can then be used in untreated cells. One such method of treatment which can produce this effect is random mutagenesis (Chater and Wilde 1980); treated cells that can be transformed with foreign plasmid DNA, and in which the plasmid DNA can survive may have acquired mutations in their restriction enzyme system.

A second procedure used to overcome the restriction/modification system relies on the differential sensitivity of these two enzyme systems to heat inactivation. In many

species, the restriction system is considerably more sensitive to elevated temperature than is the modification system. Immediately before transformation, protoplasts are heated to 45°C for a short period of time. The heat labile restriction system is destroyed and upon transformation the modification enzyme will be able to alter the foriegn DNA to the host's specifications before the restriction enzyme has been resynthesized (Hunter, personal communication).

Since the discovery that *Streptomyces* spp. produce beta-lactam compounds, investigators have elucidated some of the biochemical pathways, and are now turning their attention to the genetic aspects of antibiotic production. Each *Streptomyces* sp. appears to be different with respect to plasmid content, and plasmid involvement in antibiotic production. The goal is to be able to manipulate antibiotic production by deregulation of production, or to produce new products. In order to do this, cloning of genes involved must be achieved. Possible vectors are being investigated, and requirements for transformations are being determined.

III. MATERIALS AND METHODS

III.1. BACTERIAL CULTURES

The streptomyces cultures used in this study were:

Streptomyces clavuligerus NRRL 3585

Streptomyces phaeochromogenes NCIB 8505

Streptomyces venezuelae 13s

Streptomyces venezuelae NCIB 8231

Streptomyces fradiae NCIB 8233

Streptomyces griseus NCIB 8237

Streptomyces rimosus NCIB 8229

Streptomyces lividans 1326

Streptomyces lividans 3141

Streptomyces lividans 3046

The *S. lividans* strains were obtained from Y. Aharonowitz, Tel Aviv University. The *S. venezuelae* 13s culture was obtained from L. Vining, Dalhousie University.

Escherichia coli Ess was used for the bioassay of antibiotic produced by *S. clavuligerus*. This particular strain of *E. coli* is a mutant which is very sensitive to beta-lactam antibiotics and it was obtained from A. L. Demain, Massachusetts Institute of Technology. The culture used for the clavulanic acid bioassay was *Staphylococcus aureus* N2. This *S. aureus* strain is insensitive to beta-lactam antibiotics due to the production of a beta-lactamase, but with the addition of clavulanic acid, the beta-lactamase was inhibited, rendering the *S. aureus* sensitive to penicillin.

Five plasmid bearing strains of *Escherichia coli* were used as known standards for some of the plasmid preparations. These strains were:

Escherichia coli HB101 with plasmid pBR322

Escherichia coli J53-1 with plasmid R307

Escherichia coli J53-1 with plasmid R177

Escherichia coli J53-1 with plasmid R27

Escherichia coli J53-1 with plasmid pSD114

The strains were obtained from J. Foght.

III.2. MAINTENANCE AND CULTIVATION OF BACTERIAL CULTURES

III.2.1. Maintenance of *Streptomyces* spp. cultures

A liquid nitrogen slant of *S. clavuligerus* NRRL 3585 was used to inoculate tomato-oatmeal agar plates [per litre: 20.0g tomato paste; 20.0g oatmeal pablum; 25.0g Bacto agar; adjusted to pH 6.8]. These plates were incubated at 28°C until spores were formed.

Spores were removed from the colonies on the tomato-oatmeal agar plates by scraping with a sterile spatula and suspending in distilled H_2O. Sterile glycerol was added to a final concentration of 20% (v/v). This spore suspension was then dispensed into sterile plastic screw capped tubes (NUNC Laboratories) and stored at -20°C. The viability was determined by serially diluting the thawed spore suspension in distilled H_2O and then plating 0.1 mL amounts on tomato-oatmeal agar plates. Spore stocks were found to contain 10^7 to 10^8 viable spores/mL. This same procedure was used for the maintenance and preparation of spore stocks for the other *Streptomyces* spp. used in this study.

III.2.2. Cultivation of *Streptomyces* spp. in Liquid Medium

In order to culture *S. clavuligerus* , a spore stock was thawed, and 0.5 mL was used to inoculate 25 mL of phytone seed medium [per litre, 15 g of phytone, 10 g glycerol, and 1 g yeast extract, pH 6.5] or Glycerol-YEME [per litre: yeast extract, 4.0 g; malt extract, 10 g; glycerol, 10 mL; pH 6.8] in a 125 mL flask. These broth cultures were incubated at 28°C on a New Brunswick Scientific shaker at 250

revolutions per minute, for a period of 2 to 4 days.

The *S. lividans* strains were grown in a GYEME medium [per litre glucose, 10 g; yeast extract, 4.0 g; malt extract, 10 g; pH 6.8] supplemented with 34% sucrose. The remainder of the *Streptomyces* spp. were grown in phytone seed medium. A volume of 25 mL of medium was placed in a 125 mL flask. The cultures were incubated at 28°C on a New Brunswick Scientific shaker at 250 revolutions per minute.

III.2.3. Maintenance of Other Cultures

The *E. coli* cultures for plasmid preparations were grown in 50 mL flasks of 10 mL containing brain heart infusion broth (BHI, Difco Laboratories) with 5 antibiotic impregnated discs (Becton Dickinson Sensi-Discs and Difco Dispens-o-disc) required for maintenance of the plasmids. For plasmids pBR322 and R27 tetracycline discs were added to the medium, for pR307 kanamycin discs were added, and for pR177 carbenicillin discs were added. The cultures were incubated at 250 rpm, for 16 hours at 37°C. The cultures were maintained on BHI plates on which were placed 5 of the appropriate antibiotic discs. The plates were incubated at 37°C for up to 48 hours after which they were stored at 4°C. The cultures were transferred at monthly intervals in order to ensure maintenance of their plasmids.

E. coli Ess was maintained on trypticase soy broth plates (TCS, BBL Laboratories). To obtain an overnight culture for use in a bioassay, 10 mL of TCS was inoculated with a single colony and then incubated at 37°C for about 16 hours. *S. aureus* N2 was maintained on blood agar plates. To obtain an overnight culture for bioassay, a single colony was suspended in 10 mL of nutrient broth (Difco) and incubated at 37°C for about 16 hours.

III.3. SELECTION OF A SINGLE SPORE ISOLATE OF *S. CLAVULIGERUS*

A spore suspension, prepared from tomato-oatmeal agar plates, was diluted to 10^{-7} in distilled H_2O. A volume of 0.1 mL of these dilutions was spread on

tomato-oatmeal agar plates. The plates were incubated at 28°C for twelve days. The colonies which developed on the plates typically displayed a variety of colony morphologies. This morphological variability is a common occurance in many *Streptomyces* spp. One colony representative of each type was selected and used to inoculate 25 mL of phytone seed medium. The cultures were incubated at 28°C on a shaker. After 2 days and 6 days, 1 mL of the cultures was filtered through a Whatman #1 filter by gravity. The filtrates were assayed for antibiotic production and clavulanic acid production by the bioassay method.

III.3.1. Bioassay for Antibiotic Production

The bioassay for antibiotic production was performed using TCS agar, inoculated to 2% with an overnight culture of *Escherichia coli* Ess, dispensed in 10 mL amounts in petri dishes. A sterile paper disc was placed in the centre of the plate, and 20 µL of culture filtrate was applied to the disc. The plates were incubated at 37°C for 24 hours, and the zones of inhibition of growth which resulted were compared to those obtained with standard solutions of cephalosporin C.

III.3.2. Bioassay for Clavulanic Acid Production

Filter sterilized penicillin G, obtained from Bristol-Myers, was added to TCS agar at a final concentration of 1 µg/mL. To this a 1% inoculum of an overnight culture of *Staphylococcus aureus* N2 was added, and the agar dispensed in 10 mL amounts in petri dishes. A sterile paper disc was placed in the centre of the plate and 20 µL of the culture filtrate was placed on the disc . The plates were incubated at 37°C for 24 hours, and the zones of inhibition of growth of the *S. aureus* N2 which resulted were compared to those obtained with standard solutions of clavulanic acid.

The single spore isolate showing good characteristics of spore formation and production of beta-lactam compounds was then used for the remainder of the studies.

III.4. PLASMID CURING TECHNIQUES

III.4.1. Protoplast Formation and Regeneration

III.4.1.1. Solutions and Media

All materials and solutions were sterile, and aseptic technique was observed. The method for protoplast formation and regeneration used was that of Okanishi *et al.* (1975), as modified by Thompson *et al.* (1984). When protoplasts were made of *S. clavuligerus* there were some modifications to the procedure, appropriate for this culture. L-medium referred to the solution containing lysozyme, and P-medium referred to the isotonic solution used to stabilize protoplasts, as designated by Okanishi *et al.* (1975), and Thompson *et al.* (1984).

L-medium:

sucrose	100 g
K_2SO_4	0.25 g
$MgCl_2 \cdot 6H_2O$	0.508 g
trace elements	2.0 mL
final volume	880 mL
After autoclaving add:	
0.5% KH_2PO_4	10.0 mL
3.68% $CaCl_2 \cdot 2H_2O$	10.0 mL
0.25M MOPS, pH 7.2	100.0 mL
lysozyme	1 mg/mL

P-medium:

sucrose	103 g
K_2SO_4	0.25 g
trace elements	2.0 mL
$MgCl_2 \cdot 6H_2O$	2.03 g

final volume	790 mL

After autoclaving add:

0.5%KH_2PO_4	10.0 mL
3.68%$CaCl_2 \cdot 2H_2O$	100.0 mL
0.25M MOPS, pH 7.2	100.0 mL

Trace Elements:	per litre
$ZnCl_2$	40 mg
$FeCl_3 \cdot 6H_2O$	200 mg
$CuCl_2 \cdot 2H_2O$	10 mg
$MnCl_2 \cdot 4H_2O$	10 mg
$Na_2B_4O_7 \cdot 10H_2O$	10 mg
$(NH_4)_6Mo_{24} \cdot 4H_2O$	10 mg

Regeneration Agar (R2YE):

sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2 \cdot 6H_2O$	12 g
trace elements	2.0 mL
glycerol	10.0 mL
Difco casamino acids	0.1 g
agar	22 g
L-asparagine	1.8 g
Difco yeast extract	5.0 g
final volume	810 mL

After autoclaving, add:

0.5% KH_2PO_4	10.0 mL
3.68%$CaCl_2 \cdot 2H_2O$	80 mL
0.25M MOPS, pH 7.2	100 mL

The R2YE was dispensed in 20 mL amounts in petri dishes.

III.4.1.2. Preparation of Protoplasts

Spores (0.5 mL of a spore suspension containing 10^7 to 10^8 spores/mL) of *S. clavuligerus* were used to inoculate a 125 mL flask containing 25 mL of either glycerol-YEME, or phytone seed medium. This culture was incubated at 28°C, at 250 rpm, for 40 hours, and was then used to inoculate fresh broth. The fresh broth was supplemented with 0.8% glycine, and 5mM $MgCl_2$. These cultures were incubated at 28°C, at 250 rpm, for 40 hours before being harvested. The volumes of solutions given below refer to an original culture volume of 25 mL.

Mycelia were harvested by centrifugation at 27,000x g for 15 minutes. After the supernatant was removed the pellet was resuspended in 10 mL of 0.3M sucrose. The mycelia were again collected by centrifugation but this time at 3,000x g for 15 minutes. The pellet was resuspended in 5 mL of 0.3M sucrose. The suspension was transferred to a Kontes glass homogenizer and homogenized briefly. The suspension was transferred back to the centrifuge tube and a further 5 mL of 0.3M sucrose was added. The mycelia were again collected by centrifugation at 1,500x g for 10 minutes. The pellet was drained and then resuspended in 4 mL of L-medium. The mycelia were incubated in L-medium at 32°C for up to 90 minutes, with gentle mixing. The formation of protoplasts was followed by using phase contrast microscopy. Once protoplast formation appeared to be complete, 5 mL of P-medium was added to the protoplast suspension and the mixture was pipetted up and down gently to further aid in the release of protoplasts. The suspension was then filtered through sterile cotton wool to remove long mycelia that remained. The protoplasts were then centrifuged, at room temperature, at 1,500x g for 7 minutes. The protoplasts were resuspended in 10 mL of P-medium. At this point, a portion of the protoplasts were diluted in P-medium and counted in an AO Scientific Instruments Bright-Line hemocytometer. The remainder of the protoplasts were again centrifuged at 1,500x g, at 22°C, for 7 minutes. The pelleted protoplasts

were then resuspended in an appropriate volume of P-medium, and either used immediately or stored at -70°C.

III.4.1.3. Protoplast Regeneration

Protoplasts of *S. clavuligerus* were serially diluted in both P-medium and distilled H_2O to distinguish protoplasts from non-protoplasted units. A volume of 0.05 mL of the dilutions was spread, using a glass spreader, on R2YE agar and incubated 24 hours at 22°C. Following this intial time of incubation, plates were placed at 28°C for the remainder of the incubation period. The actual number of protoplasts which regenerated was determined by taking the total number of colonies, arising on the R2YE media, from the P-medium dilution series and subtracting that arising from the H_2O dilution series. The percentage survival was calculated from the number of protoplasts regenerated and the total number of protoplasts counted in the hemocytometer.

III.4.2. Growth at Elevated Temperatures

Growth at temperatures, elevated by 5°C over the optimum growth temperature, can be used as a curing technique (Carlton and Brown 1981). *S. clavuligerus* spores (0.5 mL of a spore suspension of 10^7 to 10^8 spores/mL) were used to inoculate 25 mL of glycerol-YEME broth in a 125 mL flask. The culture was incubated at 28°C, at 250 rpm, for two days. The culture was then used as an inoculum for fresh medium. One flask of glycerol-YEME was placed at 28°C, and three other flasks were incubated at temperatures of 33°C, 34°C, and 35°C. After 2 days incubation, the cultures were transferred to fresh media and incubation was continued at the same temperatures. Incubation was continued again for 2 days and again the cultures were transferred and incubation continued.

Cultures were diluted and plated on glycerol-YEME agar [glycerol-YEME broth with 2% agar]. The percent survival was determined.

III.4.3. Growth in the Presence of Novobiocin

III.4.3.1. Growth in Broth Culture

A 48 hour broth culture of *S. clavuligerus* in 25 mL of glycerol-YEME was used to inoculate flasks of 25 mL of fresh glycerol-YEME containing various concentrations of novobiocin. The flasks were incubated at 28°C, shaking at 250 rpm. After 3 days, the cultures were observed microscopically and macroscopically to determine the concentration of novobiocin which gave partial inhibition of growth for use in curing experiments.

III.4.3.2. Growth on Solid Medium

Spores were diluted in distilled H_2O and 0.1 mL of the dilutions were spread on phytone seed agar plates containing from 0 to 5 μg/mL of novobiocin. The plates were incubated at 28°C. Colonies arising on the plates were counted, and the percentage survival was determined.

III.4.4. Testing for Changes in Antibiotic or Clavulanic Acid Production

Colonies which resulted from the various curing procedures were patched to phytone seed agar on 24.3 cm x 24.3 cm plates (NUNC Laboratories) and incubated for up to three days at 28°C. At that time the colonies were tested for their ability to produce antibiotics by the bioassay system by overlaying the plate with 100 mL of TCS agar inoculated to 2% with an overnight culture of *E. coli* Ess. Any colonies which did not give a zone of inhibition were presumed to be antibiotic non-producing colonies and these were isolated and retested.

A second method for antibiotic production testing was to pick isolated colonies onto plugs of phytone seed agar cut from a petri dish plate with a sterile cork borer (3 mm diameter). After the colony on the plug had grown, the plug was transferred to a

petri plate containing *E. coli* Ess inoculated TCS agar, and the colony was then bioassayed as described before. In a similar manner, clavulanic acid production was tested by transferring the agar plugs to *S. aureus* N2 inoculated nutrient agar containing penicillin G as described previously.

III.5. PLASMID ISOLATION

III.5.1. Alkaline Sodium Dodecyl Sulfate (SDS) Procedures

III.5.1.1. Birnboim and Doly

This procedure for plasmid isolation was a modification of the method used by Birnboim and Doly (1979). The solutions used were prepared using ultra pure water (obtained with a Millipore Milli-Q system, hence referred to as Milli-Q water), and were sterilized before use. The procedure used either colonies from plate cultures of *Streptomyces sp.* or *E. coli* , or 0.1 mL- 0.5 mL volumes of broth cultures. When using broth cultures for the streptomycetes, glycine was added to the medium before inoculation so as to sensitize the mycelia to lysis, and the cultures were incubated for a period of 48 hours. If *S. clavuligerus* was being used, 0.8% glycine was the final concentration; for *S. lividans*, 0.5% glycine was used, and for the remainder of the streptomycetes, 1.0% glycine was used. The cells were suspended in 100 µL of solution I [50mM glucose, 10mM EDTA, and 25mM Tris-HCl, pH 8.0]. To this 5 µL of fresh lysozyme solution [4 mg lysozyme per 100 µL of solution I] was added; the tube was mixed and incubated for 1 hour at 37°C. Following the incubation period, 200 µL of 0.2N NaOH containing 1% SDS was added, the tube mixed by inversion and incubated at 22°C for exactly 6 minutes. After this, 150 µL of 3M Na acetate, adjusted to pH 4.8 with glacial acetic acid was added, the tube mixed, and then held at 0°C for 1 hour. The tube was centrifuged in an Eppendorf microfuge for 5 minutes after which 0.4 mL of the supernatant was transferred to a clean tube. The DNA was precipitated by the addition

of 0.11 volumes of 3M sodium acetate and 2 volumes of cold 95% ethanol and placed at -20°C for 2 to 16 hours, centrifuged and resuspended in 100 µL of 0.1M Na acetate containing 50mM Tris-HCl, pH 8.0, and precipitated with ethanol a second time. Following this the DNA pellet was resuspended in 20 µL of Milli-Q water, to which 5 µL of 5x sample buffer [25% sucrose, 5mM Na acetate, 0.05% bromophenol blue, 0.1% SDS] was added. In some cases the 5x sample buffer was replaced with a Ficoll sample buffer [16% Ficoll 400,000 Pharmacia, 50mM EDTA, 0.05% bromophenol blue or orange G].

III.5.1.2. Kieser's Alkaline Lysis Procedure

This alternative procedure for plasmid isolation (Kieser *et al.* 1982), was used as a screening procedure for the detection of plasmids in transformed cultures, and was carried out in 1.5 mL Eppendorf tubes. The source of mycelia was a 10 mL broth culture, appropriate for the species of streptomyces being tested. A volume of 1.5 mL of culture was centrifuged in a microfuge to pellet the mycelia, and the supernatant was discarded. The pellet was resuspended in 500 µL of 25mM Tris-HCl (pH 8.0), 25mM EDTA, 0.3M sucrose, and 0.02% orange G dye, containing 2 mg lysozyme/mL. This was incubated at 37°C for 30 minutes. After the incubation, the tube was mixed and 250 µL of 0.3M NaOH containing 2% SDS was added. This was mixed completely and then incubated at 70°C for 15 minutes. The tube was cooled to 22°C, and 80 µL of acid phenol/chloroform [5 g phenol, 5 mL chloroform, 1 mL H_2O, and 5 mg 8-hydroxyquinoline] was added. The tubes were mixed thoroughly and then centrifuged to separate the phases. The samples were then subjected to agarose gel electrophoresis.

III.5.2. Neutral Lysis Procedures

III.5.2.1. Westpheling

This procedure follows that of Westpheling (1980). A 25 mL culture of *S. clavuligerus* grown in glycerol-YEME or phytone seed medium, supplemented with 0.8% glycine and 5mM $MgCl_2$, was harvested by centrifugation at 27,000x g for 15 minutes. The pellet of mycelia was resuspended in 10 mL of TE buffer [10mMTris-HCl, pH 8.0; 1mM EDTA] containing 15% sucrose. This was then centrifuged at 27,000x g for 10 minutes; the pellet was resuspended in 4 mL 0.05M

Tris-HCl, pH 8.0, containing 15% sucrose. One millilitre of fresh lysozyme solution at 5 mg lysozyme/1 mL of 0.25M Tris-HCl, pH 8.0 was then added and the suspension was incubated at 30°C, with continuous gentle mixing, until the appearance became "fluffy", but before complete lysis had occured. After the incubation, 2 mL of 0.25M EDTA, pH 8.0, and 3 mL of 20% SDS were added and the suspension was allowed to clear. To this, 3 mL of 5M NaCl was added; the suspension was left at 4°C for 2 to 16 hours. This was then centrifuged at 30,000x g for 20 minutes at 4°C. The supernatant was saved, and to it was added 4 mL of sterile 50% polyethylene glycol 6000 (PEG) and this was left at 4°C for 2 to 16 hours. The precipitated DNA was collected by gentle centrifugation, 1,500x g for 5 minutes, and then resuspended in up to 1 mL of sterile TE buffer.

III.5.2.2. Modified Neutral Lysis Procedure

The Westpheling procedure for plasmid isolation was modified in order to decrease the amount of time required which in turn would minimize the degradation of the DNA isolated. The mycelia were harvested by centrifugation from a 25 mL broth culture, and washed in TE plus 15% sucrose as with the original procedure. The pellet was resuspended in 5 mL of 0.05M Tris-HCl, pH 8.0, plus 15% sucrose, and again 1 mL of lysozyme solution was added, but at a much higher concentration, 150 mg lysozyme/mL of 0.25 M Tris-HCl, pH 8.0. The suspension was incubated at 30°C, with continous agitation, for 7 minutes. Following this, EDTA and SDS were added as

in the original procedure. Three millilitres of 5M NaCl was added, and the tube was placed on ice for 10 minutes, by which time precipitation of denatured chromosomal DNA and cell debris was complete. To obtain a firm pellet, the tube was centrifuged at 48,000x g for 60 minutes. The supernatant was then extracted with equal volumes of TE buffer equilibrated phenol, mixed and then centrifuged at 1,500x g for 10 minutes to separate the phases. The aqueous phase was extracted with an equal volume of phenol/chloroform [50/50, v/v] in the same manner. The DNA was precipitated with ethanol from the aqueous phase as described previously. The precipitated DNA was collected by centrifugation at 1,500x g for 5 minutes and resuspended in up to 1 mL of TE buffer.

III.5.2.3. Large Scale Plasmid Isolation

The Westpheling neutral lysis procedure was scaled up in order to process 500 mL amounts of *Streptomyces* spp. culture. The culture medium used varied according to the particular species as indicated earlier. For example, spores of *S. lividans* were used to inoculate 25 mL of GYEME plus 34% sucrose. The flask was incubated at 28°C, at 250 rpm, for 40 hours, at which time the culture was subcultured at 2% v/v into 5 flasks of 100 mL of the same media, supplemented with glycine at 0.5%, and 5mM $MgCl_2$. These flasks were incubated at 28°C, at 250 rpm, for a further 40 hours. The mycelia were harvested by centrifugation and processed according to the Westpheling neutral lysis procedure as described previously. The pellet of DNA obtained after PEG precipitation was dissolved in 5 to 10 mL of TE buffer. To this, 1.05 g CsCl was added for each millilitre of DNA solution. The suspension was then centrifuged at 3,000x g for 5 minutes in order to remove lipids. The refractive index was adjusted to 1.3945 and ethidium bromide was added to a final concentration of 10 µg/mL. This was centrifuged at 90,000x g, at 20°C, for 40 hours. Upon completion of the centrifugation, the tubes

were examined with a Cole-Parmer hand-held UV illuminator for evidence of ethidium bromide stained bands of DNA. The lower band was removed by puncturing the side of the tube with a 22 gauge needle and removing the band with a syringe.

The collected bands were placed in silanized glass tubes, and the ethidium bromide was removed by repeated extraction with an equal volume of H_2O equilibrated n-butanol. The CsCl was removed by overnight dialysis against 0.1SSC [1SSC: 0.15M NaCl, 0.015M Na citrate]. If it was necessary to concentrate the DNA it was ethanol precipitated as described previously. The amount of DNA present was determined by reading the absorbance of the DNA solution at 260 nm, knowing that 50 µg DNA/mL gives an A_{260} of 1.

III.5.3. Isolation of Linear Extrachromosomal DNA

Linear extrachromosomal DNA can be isolated using a modification of the procedure of Fishmann and Hershberger (1984), as developed by Keen (1985). Spores of *S. clavuligerus* were used to inoculate TCS broth containing 1% glycerol. The culture was incubated, for 40 hours, at 28°C and then used to inoculate 100 mL of the same medium. This culture was incubated for 24 hours and then used for DNA isolation . The mycelia were collected by centrifugation and each 10 g wet weight of mycelia were resuspended in 100 mL of TES [10mM Tris-HCl, 1.0mM EDTA, and 25% sucrose, pH 8.0]. Fifty millilitres of 0.25M EDTA, 50 mL of a lysozyme solution [10 mg lysozyme per millilitre of TES], and 0.2 mL diethylpyrocarbonate were added to the cell suspension. This was incubated for 15 minutes at 22°C, after which proteinase K (Sigma proteinase XI) and SDS were added to give final concentrations of 50 µg/mL and 10 mg/mL respectively. The mixture was incubated at 55°C for 30 minutes. Following this, 20 mL of 5M NaCl was added and the mixture was then incubated on ice for 1 to 3 hours. This was then centrifuged at 42,000x g for 45 minutes. The DNA was

precipitated from the supernatant with 0.64 volumes of isopropyl alcohol at -20°C, and collected by centrifugation at 3,000x g for 20 minutes (Keen 1985). The DNA pellet was resuspended in TE buffer.

The linear plasmid was separated from the chromosomal DNA by the use of preparative sucrose gradient centrifugation. Gradients were prepared using a conical chambered gradient maker with 6.5 mL of 40% sucrose and 6.9 mL of 10% sucrose solutions in 20mM Tris-HCl (pH 8.0), and 1M NaCl. Once the gradients were prepared, they were placed at 4°C for 2 hours and then 0.2 mL of DNA solution was carefully layered on the top of the gradients. The gradients were balanced and then centrifuged at 175,000x g for 20 hours. After the centrifugation was completed, the gradients were fractionated by repeatedly removing aliquots of 0.4 mL from the top, using an Eppendorf pipette. The DNA in the fractions was precipitated with 95% ethanol and stored at -20°C.

III.6. RESTRICTION ENDONUCLEASE DIGESTIONS

Enzymes used for restriction digests were *Bgl* II (BRL), *Sst* II (BRL), *Sal* I (BRL, lyphozyme), *Sma* I (BRL, lyphozyme), *Cla* I (Boehringer Mannheim), *Pst* I (BRL), *Bst* EII (BioLabs), *Bam* HI (BioLabs), and *Hind* III (BRL). The restriction enzymes used were generously supplied by K. L. Roy. The lambda DNA used as a standard for most digestions was purchased from P.L. Biochemicals. The restriction enzyme buffers and temperatures of incubation were those recommended by the manufacturer. As a rule, 10 units of an enzyme was required to digest 1 µg of *Streptomyces* spp. DNA with incubation times of 2 to 3 hours.

III.7. ELECTROPHORESIS AND PHOTOGRAPHY

Electrophoresis in agarose gels was used to separate DNA. Agarose (SeaKem Type LE) was dissolved in TEA buffer [20mM Tris-HCl (pH 8.0), 2mM EDTA, and 50mM sodium acetate] and the concentration of the agarose varied from 0.7% - 1.25%

w/v depending on the DNA preparation. The voltage for electrophoresis was up to 6 volts per centimetre of the length of the gel. Once electrophoresis was completed, the gel was placed in a tray, and stained with 10 μg/mL of ethidium bromide in H_2O, for 30 minutes. After staining, the gel was rinsed in either distilled H_2O or 0.01M $MgCl_2$ to remove excess ethidium bromide. Gels were photographed with Ilford negative film, or Polaroid type 667 and type 665 film, using UV light at 300 nm and a Kodak Wratten gelatin filter number 23A.

III.8. TRANSFORMATION STUDIES

III.8.1. Solutions and Media

Transformation studies used the same solutions for protoplast formation and regeneration as described earlier. In addition, T-medium was used.

T-Medium:

sucrose	0.25 g
K_2SO_4	0.0025 g
PEG 1000	2.5 g
$MgCl_2 \cdot 6H_2O$	0.02 g
trace elements	0.02 mL

Adjust volume to 7.9 mL with H_2O,
and after autoclaving add:

14.72% $CaCl_2 \cdot 2H_2O$	1.0 mL
0.5% KH_2PO_4	0.1 mL
0.5M Tris-maleic acid, pH 8.0	1.0 mL

III.8.2. Transformation of *S. lividans*

The procedure for the transformation of *S. lividans* with plasmid vectors pIJ702 and pIJ303 had already been established (Kieser *et al.* 1982). Plasmid DNA for transformation studies was prepared by the large scale neutral lysis procedure as described earlier. Protoplasts, about 4 x 10^9 protoplasts per transformation, of *S.*

lividans were centrifuged at 1,500x g to sediment and resuspended in a minimal volume, using the residual liquid in the centrifuge tube. Ten microlitres of 10.3% sucrose was added to the protoplasts and then 10 µL of plasmid DNA solution containing up to 1 µg of DNA. The control performed for any transformation experiment was the addition of 10 µL of TE buffer (no DNA). Five hundred microlitres of T-medium was added to all the tubes.

The tubes were mixed gently for 30 seconds, and then the transformation reaction was terminated by the addition of 5 mL of P-medium. The protoplasts were pelleted by centrifugation at 1,500x g for 7 minutes, and then resuspended in 0.5 mL of P-medium. The protoplasts were counted using the hemocytometer as described earlier, and the appropriate dilutions were made in both P-medium and distilled H_2O. A volume of 50 µL of the dilutions was spread gently on R2YE and the plates were then left at 22°C overnight.

III.8.3. Transformation of *S. clavuligerus*

The transformation of *S. clavuligerus*, with the plasmid vector pIJ702, was performed in the same manner as described above. Initially, transformations were performed using protoplasts obtained from an unaltered culture of *S. clavuligerus*, but these transformations were unsuccessful. The inability to transform *S. clavuligerus* with pIJ702 was postulated to be due to a restriction barrier, since pIJ702 had been isolated from a *S. lividans* culture. Two different methods were used to get around the difficulty observed. In the first method, protoplasts were prepared from an *S. clavuligerus* culture which was cultivated as described previously except that the spores used as an inoculum had been exposed to N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at 1 mg/mL for 1 hour. Protoplasts derived from this culture were transformed as described earlier. An NTG mutagenized culture was used for obtaining protoplasts

because the mutagenic treatment might have generated a mutant with a defective restriction system. The second method involved making protoplasts of a normal *S. clavuligerus* culture, but then, before the actual transformation steps, heating the protoplasts to 45°C for a period of 5, 10, or 15 minutes. The heat shock treatment should inactivate the restriction enzyme, allowing for transformation with pIJ702, and modification of the plasmid before restriction activity returned (I. Hunter, personal communication). After the heat shock, the protoplasts were transformed as usual.

III.8.4. Detection of Transformants

The R2YE plates from the transformation of *S. lividans* , were overlayed with 5 mL of thiostrepton-containing soft agar [R2YE with 0.7% agar and 25 μg/mL thiostrepton], for a 20 mL plate, after 24 hours incubation. Thiostrepton was generously provided by Dr. S. Lucitania, Squibb Laboratories. The plates were incubated until colonies appeared; these colonies were thiostrepton resistant, and therefore contained the plasmid pIJ702. With *S. clavuligerus* the R2YE plates were overlayed after 48 hours incubation. Again, any colonies that appeared were thiostrepton resistant transformants.

III.9. HYBRIDIZATION STUDIES

III.9.1. Southern Transfer

III.9.1.1. Solutions

Denaturing solution	1.5M NaCl 0.5M NaOH
Neutralizing solution	3M NaOAc pH 5.5 with glacial acetic acid
20x SSPE	3M NaCl 0.2M $NaH_2PO_4 \cdot H_2O$ 0.2M Na_2EDTA pH 7.4 with NaOH

III.9.1.2. Transfer of DNA

DNA contained in agarose gels was transferred to nitrocellulose (Schleicher and Schull BA85) as described in the literature (Southern 1975; Hopwood *et al.* 1985). Briefly, DNA containing agarose gels were soaked in 2 changes of 0.4M HCl for 7 minutes to depurinate the DNA fragments. This treatment enhances the transfer of DNA fragments. The DNA was then denatured for 60 minutes, and then neutralized for 60 minutes by soaking the gel in the appropriate solutions. Nitrocellulose, presoaked in Milli-Q H_2O, was placed on top of the gel in the Southern transfer apparatus. The transfer buffer, used as the medium to transfer the DNA from the agarose gel to the nitrocellulose, was 20x SSPE. The transfer was allowed to continue overnight. The nitrocellulose was then washed in 2x SSPE for about 5 minutes, and air dried. It was then baked at 70°C to 80°C, under vacuum, for 2 hours.

III.9.2. Nick Translation

III.9.2.1. Materials and Solutions

The Klenow fragment of *E. coli* DNA polymerase I was purchased from P.L. Biochemicals and DNase was purchased from Sigma. Both were generously supplied by K. L. Roy. The [α-^{32}P]dATP and [α-^{32}P]dCTP were purchased from Amersham. Both had a specific activity of about 3,000 Ci/nmole, with a radioactivity concentration of 10 mCi/mL. The deoxynucleoside triphosphates were purchased from P.L. Biochemicals.

Nick-translation buffer (4x):

0.2M Tris-HCl pH 7.8
0.02M $MgCl_2$
0.01M dithiothreitol
0.2 µg/mL bovine serum albumin (BSA)

III.9.2.2. Nick-translation Procedure

The reaction mixture was as follows:

5 µL 4x nick translation buffer
1 µL [α-^{32}P]-dATP (10mCi/mL)
1 µL of each of 0.5mM dCTP, dGTP, dTTP
0.4 µg plasmid DNA
0.025 µg/mL DNase
0.5 units/ µL polymerase I

The total volume of the reaction mixture was 20 µL and it was incubated at 15°C for 60 minutes. The reaction was stopped by the addition of 1 µL of 0.5M EDTA, pH 8.0. To this, 1 µL of 2% bromophenol blue was added, and the mixture was then passed through a small Sephadex G50 Superfine column (Pharmacia P-L Biochemicals, Inc.), equilibrated in 10mM Tris-HCl (pH 8.0), 1mM EDTA, and 0.1% SDS. The column chromatography separated the labelled plasmid DNA from the α-^{32}P dATP. In one reaction [α-^{32}P]dCTP was used in place of [α-^{32}P]dATP.

III.9.3. Hybridization

III.9.3.1. Materials and Solutions

The SSPE solution referred to here was the same as that referred to earlier. The non-homologous DNA, supplied by K. Roy, was isolated from *E. coli* C_4F_1, and used at 150 µg/mL.

Denhardt's solution (20x):

1.0 g Ficoll
1.0 g polyvinyl pyrollidone
1.0 g BSA
in 250 mL of 3x SSPE

Prehybridization solution (final concentrations):

3x SSPE
4X Denhardt's solution
150 µg/mL non-homologous DNA

Hybridization solution (final concentrations):
3x SSPE
4x Denhardt's solution

III.9.3.2. Hybridization Procedure

The nitrocellulose was placed in a heat sealed plastic bag with prehybridization solution. This was incubated at 65°C for 1 hour. After the incubation, the prehybridization solution was removed, and replaced with the hybridization solution. The nick-translated plasmid DNA, about 0.4 μg, was denatured by heating at 90°C for 5 minutes and then cooled and added to the hybridization solution. This was incubated at 65°C for 16 hours. The solution was removed, and the filter was washed in two changes of 2x SSPE containing 0.1% SDS for 30 minutes at 70°C, and then the nitrocellulose was air dried.

III.9.4. Autoradiography

Autoradiograms of the hybridized nitrocellulose filters were prepared. The nitrocellulose was placed in an X-ray film cassette and covered with Saran Wrap. In the dark room, a sheet of X-ray film (X-Omat AR film) was placed on top of the nitrocellulose and the cassette was sealed. The film was left on the nitrocellulose for 24 hours or more at -70°C, and then developed according to the manufacturer's directions.

IV. RESULTS AND DISCUSSION

IV.1. SELECTION OF A SINGLE SPORE ISOLATE

Streptomyces spp. show great morphological variation, and so it is important to select a single spore isolate. The purpose of this survey was to select a single spore isolate to be used, through the remainder of the research work, which was typical of *S. clavuligerus* as described in the literature. The characteristics sought were those of good sporulation, antibiotic production, and clavulanic acid production.

The single spore isolates were obtained by diluting a suspension of *S. clavuligerus* spores and plating the dilutions on tomato-oatmeal agar. Each colony was assumed to have arisen from a single spore. Tomato-oatmeal agar was used since it is one of the few growth media which supports good sporulation of *S. clavuligerus*. The colony morphology of *S. clavuligerus* on tomato-oatmeal agar includes creamy beige substrate mycelium, powdery white aerial mycelium, and gray-green spores (Higgens and Kastner 1971). After twelve days incubation the various colony morphologies were identified, and colonies were grouped by their similarities. A representative of each colony type was then selected to inoculate phytone seed medium to follow antibiotic and clavulanic acid production (Table 1). Ten different colony types were observed, and considerable variation was noted in the antibiotic and clavulanic acid production abilities of the various colony types. No clear relationship was seen between sporulation ability and antibiotic and clavulanic acid production, but colony type 10 was the most predominant colony type, and showed good sporulation and production characteristics. This colony was used to inoculate additional tomato-oatmeal agar plates and the resulting spores were harvested and stored at -20°C as glycerol stocks for use in the remainder of the studies. Antibiotic and clavulanic acid were periodically tested for to ensure that the isolate selected remained stable.

Table 1: Characterization of Single Spore Isolates

Colony Type	Appearance	Proportion of total colonies (%)	Antibiotic[a] Production (μg/mL) 48h	144h	Clavulanic Acid[b] Production (μg/mL) 48h	144h
1	7mm diameter, aerial mycelia, heavy with spores	18	11.5	6.1	3.0	9.0
2	6mm, little aerial mycelia, no spores	2	7.3	11.5	2.2	0
3	6mm, little aerial mycelia, few spores	9	0	9.1	0	0
4	6mm, aerial mycelia, few spores at centre of colony, but radiating rings of spores	7	11.5	11.5	7.1	7.8
5	8mm, flat, no spores some aerial mycelia	7	11.5	9.1	18.2	5.4
6	6mm, mostly substrate mycelia, ring of aerial mycelia, no spores	5	11.5	10.2	16.5	4.9
7	7.5mm, edge shows substrate mycelia aerial mycelia, no spores	7	11.5	15.0	13.5	8.5
8	7mm, radiating ridges of aerial mycelia, no spores	2	15.2	8.1	50.0	6.6
9	5mm, mostly substrate mycelia, few spores	11	9.1	8.2	13.5	7.3
10	9mm,heavy with aerial mycelia, many spores	32	11.5	16.8	37.0	11.5

a. Samples of culture filtrate (20 μL) were bioassayed using *E. coli* Ess as the indicator organism. Zones of inhibition were compared to those obtained from known amounts of cephalosporin C. Antibiotic production is expressed as μg/mL cephalosporin C equivalents.

b. Details as in a. except indicator organism was *S. aureus* N2 as clavulanic acid was being measured.

IV.2. INVESTIGATION OF PLASMID INVOLVEMENT IN ANTIBIOTIC PRODUCTION BY *STREPTOMYCES CLAVULIGERUS*

Plasmids have been found to be involved in antibiotic production in some *Streptomyces* spp. (Kirby and Hopwood 1977). Several approaches can be used to investigate the potential significance of plasmids in production of antibiotics. One is to correlate changes in antibiotic production with treatments known to promote plasmid loss, called plasmid curing. Another is to directly isolate and visualize plasmid DNA. These procedures have limitations as previously mentioned, so some combination of these approaches is presented.

IV.2.1. Curing

Since it is not possible to readily isolate all *Streptomyces* spp.plasmids, an alternative approach is to correlate the presence of a plasmid with a phenotype, or the absence of the plasmid with a loss of that phenotype. With curing techniques, a plasmid may be lost, and so following a curing procedure, isolates obtained must be tested for a loss of the characteristic of interest. Curing techniques used were protoplast formation and regeneration, growth at elevated temperatures and growth in the presence of acridine orange, or novobiocin.

IV.2.1.1. Protoplast Formation and Regeneration

Protoplast formation and regeneration has been a successful method for the curing a cell of plasmids (Hopwood 1981). *S. clavuligerus* was grown in the presence of glycine. The mycelia were exposed to lysozyme, in an isotonic medium, to remove the cell wall, and then the protoplasts were spread on R2YE regeneration agar, as described in Materials and Methods.

i) Determination of Glycine Concentration

Protoplast formation of *Streptomyces* spp. typically requires that glycine be

included in the growth medium in order to sensitize the cells to lysozyme so that they will convert to protoplasts more easily. The most suitable concentration of glycine is that which gives partial inhibition of growth. For *S. phaeochromogenes* has been reported that 1% glycine is most suitable and for *S. lividans* 0.5% glycine is optimal, so considerable variation is observed among various *Streptomyces* spp. (Kieser *et al.* 1982). The percentage of glycine (w/v) to be used for sensitizing *S. clavuligerus* was determined by observation of cultures containing various amounts of glycine.

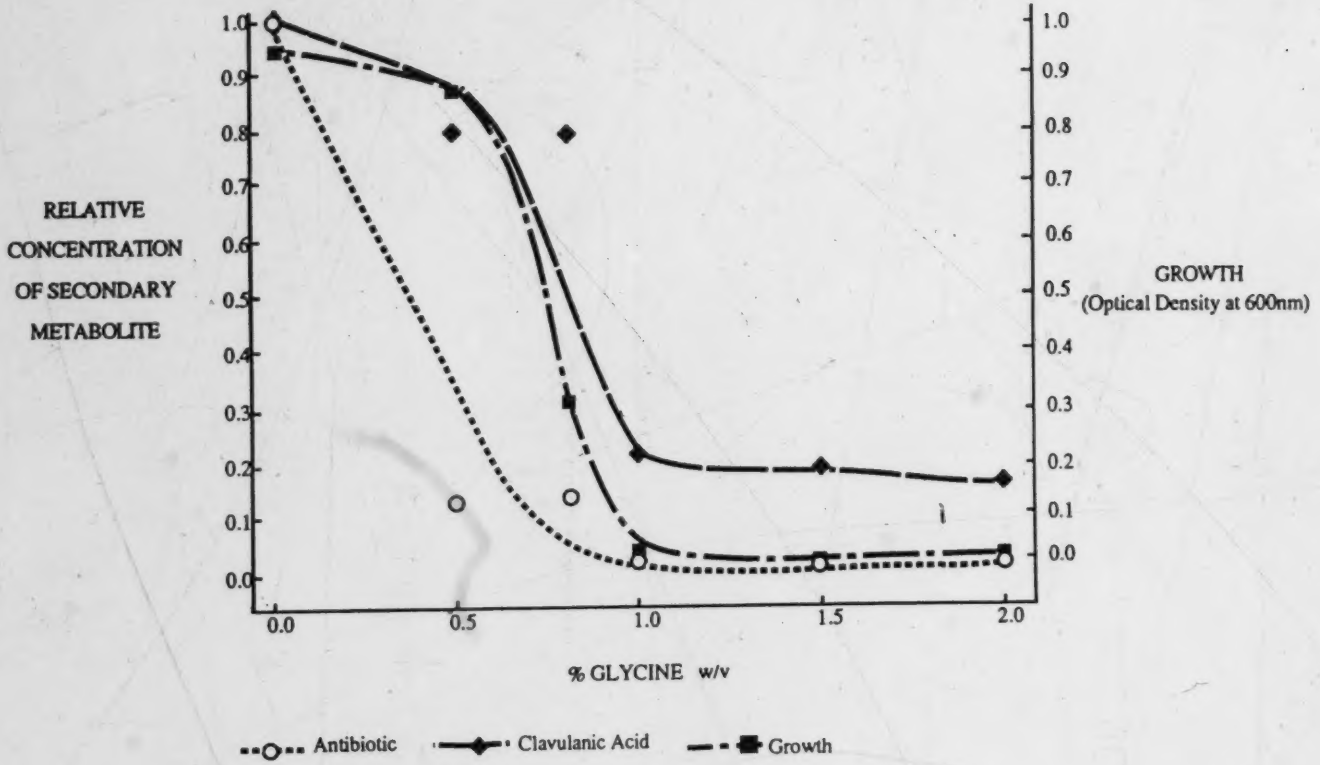
Cultures of *S. clavuligerus* were prepared in glycerol-YEME medium with glycine added at various concentrations from 0% to 2.0% (w/v). The cultures were incubated for a period of 24 hours and then the optical density at 600nm, clavulanic acid production and antibiotic production of the cultures were measured (Figure 1).

As can be seen, the control culture, containing no glycine, reached the highest optical density and the culture containing 0.5% glycine showed little growth inhibition relative to the control. The culture containing 0.8% glycine showed the desired partial guowth inhibition, while cultures containing 1% glycine or more showed essentially no growth. Antibiotic production appeared to be more sensitive to glycine than did growth. The control culture produced the greatest amount of antibiotic, but 0.5% glycine, while having little effect on growth, markedly inhibited antibiotic production. Cultures containing glycine at 1% or higher produced no detectable antibiotic. The effect of glycine on clavulanic acid production showed the same pattern of inhibition as was seen with antibiotic production, but the inhibition was less severe. From these results, the most appropriate amount of glycine to add to the cultures is 0.8% (w/v) in order to obtain the best balance between unrestricted growth and inhibition.

ii) Protoplast Formation and Regeneration

Protoplasts were counted in a hemocytometer to estimate the total number of

Figure 1: Effect of glycine on growth and secondary metabolite production in *Streptomyces clavuligerus*



protoplasts formed. Regeneration ability of the protplasts was then measured by preparing dilution series of the protoplasts in both medium P and water. Samples of each dilution were spread on R2YE plates to allow regeneration to take place. Once the colonies had fully developed, the number of colonies which was actually due to the regeneration of protoplasts was determined as follows:

[# of colonies (medium P dilution)] - [# of colonies (water dilution)]

This determination was made knowing that both protoplasts and non-protoplasted mycelial fragments should survive the medium P dilution while only cells which were not protoplasts would survive the water dilution. As an example, the number of protoplasts formed from a culture of *S. clavuligerus* was 2.5×10^6 protoplasts/mL.

The percentage of protoplasts was then determined to be the number of protoplasts, from the first equation, over the number of colonies arising from the medium P dilution series, multiplied by one hundred:

$$\frac{\text{\# of protoplasts}}{\text{\# of colonies (medium P dilution)}} \times 100$$

The percent of protoplasts was used as an indication of the efficiency of the protoplast formation. The higher the percentage of protoplasts, the fewer the number of non-protoplasted fragments of mycelia. From the example above, the percent protoplasts was 99. The percent protoplasts formed was consistantly above 95%.

The percent survival of protoplasts was a reflection of whether or not the protoplasts had regenerated successfully. It was determined in the following manner:

$$\frac{\text{\# of protoplasts (regenerated)}}{\text{\# of protoplasts (total count)}} \times 100$$

Although protoplasts were diluted in osmotically supported medium P prior to plating on R2YE, the percent survival of protoplasts was always low and rarely exceeded 1%. To continue with the example given, the number of protoplasts from the total count was 3.1×10^8 protoplasts/mL and so the percent survival was 0.9%. Although this number was low, there was still 10^6 viable protoplasts. If the percent survival approached 1%, then regeneration was considered to have been successful. The percent survival was generally a reflection of how easily the culture formed protoplasts. In general, if the culture to be used for protoplast formation contained clumpy cells, or had grown slowly, prolonged incubation with lysozyme would be required to form the protoplasts. This prolonged incubation lowered the percent survival of the protoplasts formed.

In view of the variability in protoplast preparations, the regeneration of the protoplasts was checked before using protoplasts in any experiment. Since protoplasts could be stored at -70°C for several months with no loss of viability, this proved to be helpful in avoiding variation of protoplasts, particularily in transformation experiments.

iii) Curing

Cells of *S. clavuligerus* were protoplasted and, once the regenerated colonies had grown, they were tested for their ability to produce antibiotic by bioassay. Each isolate which was not able to produce antibiotic was retested a number of times, in order to ensure that the isolate would not regain its capability of producing antibiotics. Table 2 shows that a total of 905 colonies were tested, and with the first screen, 5.2% of the colonies did not produce antibiotic. These non-producing isolates were retested and on the second screen, of the original 905 colonies, only 1.2% did not produce antibiotics. After this second screen, it was noticed that the non-producing colonies also appeared to have no aerial mycelia, an appearance which was called "bald". Again these colonies were retested, and on this final screen, there were no isolates which would not produce

Table 2: Protoplast Formation and Regeneration as a method of plasmid curing. Regenerating protoplasts on R2YE plates were tested for their ability to produce antibiotics.

Total Colonies Tested	Antibiotic Negative Colonies (%)		
	First Screen	Second Screen	Final Scre[illegible]
905	5.2	1.2	0

antibiotic. It should also be noted that these colonies started to regain aerial mycelia, leading to a loss in their "baldness".

IV.2.1.2. Growth in The Presence of Novobiocin

Novobiocin has also been used successfully as a curing agent (Carlton and Brown 1981). To have an effect, novobiocin must be present in the culture medium at a high enough concentration, to cause partial growth inhibition, but not so high as to seriously inhibit, and alter the growth of *S. clavuligerus*. For this reason, it was necessary to grow *S. clavuligerus* in medium with different concentrations of novobiocin. Spores of *S. clavuligerus* were used to inoculate phytone seed medium, and after 48h growth, the culture was used to inoculate the same medium containing novobiocin, as described in Materials and Methods. After growth in the presence of novobiocin, the cultures were then plated on medium suitable for antibiotic bioassay. The number of colonies on the plates was compared to the number of colonies arising on plates from a culture which had not grown with the presence of novobiocin, giving the percent survival. Two different concentrations of novobiocin were used, 0.01 μg/mL and 0.1 μg/mL, each of which resulted in approximately the same rate of survival (Table 3). Two hundred and two colonies which had been exposed to novobiocin at 0.01 μg/mL were tested; all were capable of producing antibiotic. Three hundred and seventy colonies which had been exposed to novobiocin at 0.1 μg/mL were also tested, but in this case 8.6% of the colonies did not produce antibiotic. The non-producers were retested for antibiotic production and of the original number of colonies, 7.0% were not capable of antibiotic production. On the final screen, however, all of the colonies tested were capable of producing antibiotic. Again, initial observations of the high loss of antibiotic producing capabilities suggested that plasmid loss might be occurring.

Table 3: Novobiocin as a method for plasmid curing.

S. clavuligerus was grown at two different concentrations of novobiocin in order to obtain "cured" strains. Isolates were tested for antibiotic production.

Concentration of Novobiocin	% survival	Colonies tested	Antibiotic Negative Colonies (%)		
			1st	2nd	Final
				Screens	
0.01 mg/mL	51.3	202	0		
0.1 mg/mL	52.0	370	8.6	7.0	0

However, the subsequent return to antibiotic production was inconsistent with that conclusion.

IV.2.1.3. Growth at Elevated Temperatures

Growth at elevated temperatures has been used as a curing technique (Carlton and Brown 1981). Growth of *S. clavuligerus* at elevated temperatures was difficult in that the culture was very sensitive to temperature change. Temperatures of 33°C, 34°C, and 35°C were tested, but the cultures would only grow at the lower temperatures, and not at 35°C. Growth was an "all or none" phenomenon, and no partial inhibition of growth could be achieved. It was also very difficult to obtain a smooth broth culture; at elevated temperatures the cultures would be clumped, even when glass beads were added to the culture flask. This made it more difficult to moniter the pattern of growth.

Once a culture had grown at an elevated temperature, it was subcultured and grown at that same temperature twice before plating and testing the isolated colonies for the ability to produce antibiotic. It was found that of the 100 colonies tested, all were able to produce anitibiotic.

IV.2.1.4. Growth in the Presence of Acriflavin

Acriflavin not only acts as a curing agent, but also as a mutagen(Carlton and Brown 1981). It is important, therefore, to work with the proper concentration of acriflavin, in order to cause plasmid curing, and not mutagenesis; a lower concentration will result in curing. *S. clavuligerus* was grown first in phytone seed medium, without acriflavin for 48h, and then transfered to phytone seed medium in which acriflavin was incorporated into the medium at 1 μg/mL. This concentration gave partial inhibition of growth. Of the 100 colonies tested, all were capable of producing antibiotic.

IV.2.1.5. Discussion

For each of the curing methods used, no stable antibiotic negative isolates were

obtained. The few isolates which initially appeared to be nonproducers of antibiotic could have required a recovery period from the curing procedure before they were able again to produce antibiotic. It is possible that there is a relationship between the transient antibiotic nonproducing characteristic and the "baldness" of these isolates, since this correlation has also been noted in other *Streptomyces* spp. (Piret and Chater 1985). The curing techniques used to demonstrate the possible invovement of plasmids in the regulation or production of antibiotics did not indicate that plasmids were involved, but were sufficiently inconclusive to warrant further investigation.

IV.2.2. PLASMID ISOLATIONS

To investigate further any possible role of plasmids in antibiotic production, attempts were made to demonstrate physically the presence of a plasmid. Published reports describe a variety of procedures for the isolation of plasmids, and two general procedures suitable for use with *Streptomyces* spp. were followed: alkaline-SDS (Birnboim and Doly 1979; Ahmed and Vining 1980) and neutral lysis (Westpheling 1980). The techniques for plasmid isolation were also applied to plasmid bearing *E. coli* strains and *Streptomyces* spp. as controls where appropriate.

IV.2.2.1. Alkaline Sodium Dodecyl Sulfate Procedure

This procedure was selected as it had been used successfully to identify plasmids in *S. venezuelae* (Ahmed and Vining 1980). The separation of plasmid from the chromosomal DNA is based on the denaturation of the chromosomal DNA by alkaline conditions. The covalently closed circular nature of plasmid DNA enables it to renature once neutrality is restored. The procedure is particularly suitable for isolation of small plasmids. Broth cultures of three *Streptomyces* spp., including *S. clavuligerus*, and three *E. coli* strains were processed by the alkaline-SDS procedure give in Materials and Methods. Plasmid material was electrophoresed on a 0.7% agarose gel in TEA buffer

(Plate 1). The DNA from the bacteriophage λ (lane 1) was used as a marker to indicate approximately where chromosomal DNA would migrate, and also to serve as an internal check on how well the electrophoresis had progressed. The location of the λ DNA is marked with an arrow. The *E. coli* plasmids (lanes 2, 3, and 4) were used both as a control of the alkaline-SDS procedure, and as molecular weight markers. Plasmid DNA was successfully obtained from the *E. coli* strains containing pBR322 (lane 2) seen as a bright band near the bottom of the gel, pR307 (lane 3) seen as a band running just above the expected location for chromosomal DNA, and pR177 (lane 4) also seen as a band running above the expected location for chromosomal DNA. Plasmid DNA was also isolated from *S. venezuelae* (lane 7) and *S. phaeochromogenes* (lane 5). Literature reports indicate that *S. phaeochromogenes* contains a 10.8kb plasmid (Doull *et al.* 1983), and *S. venezuelae* 13s contains a 12.7kb plasmid (Ahmed and Vining 1983). Other bands of lower mobility than the covalently closed and circular plasmids are seen, and these are the linear and open circular forms of the plasmids. Despite repeated attempts, it was not possible to obtain any DNA from *S. clavuligerus* by the alkaline-SDS procedure. Microscopic examination of *S. clavuligerus* cells during the lysis stage of the plasmid preparation procedure indicated that the culture was lysing and so the inability to detect plasmid DNA could not be explained by inadequate lysis.

IV.2.2.2. Neutral Lysis

Since the alkaline-SDS plasmid isolation procedure was not successful in demonstrating plasmid DNA in *S. clavuligerus*, a neutral lysis procedure was tested. The neutral lysis procedure is less harsh, and has been suggested to be useful for isolation of larger, more fragile plasmids (Westpheling 1980).

i) Westpheling Neutral Lysis

This procedure was originally developed by Westpheling (1980) for the isolation

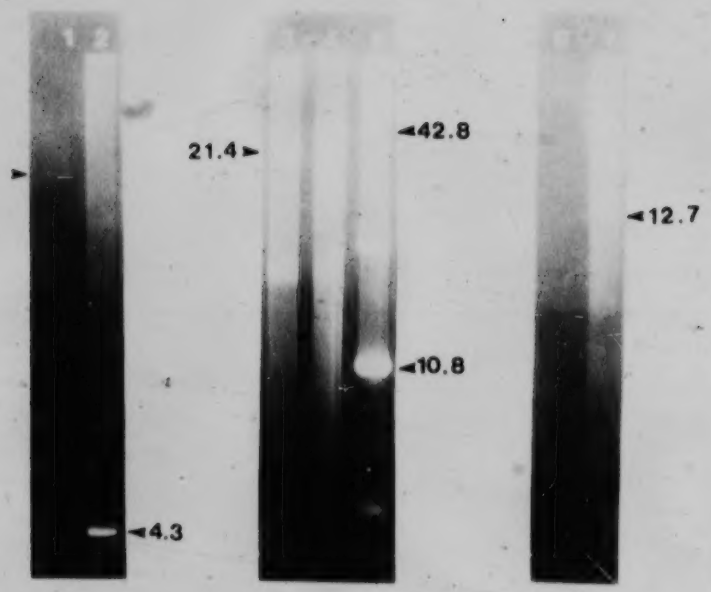


Plate 1: Agarose gel electrophoresis of plasmid DNA samples prepared by the alkaline-SDS procedure. Plasmid DNA preparations were analyzed by electrophoresis on a 0.7% agarose gel in TEA buffer. Lane 1 contains λ DNA , lanes 2, 3, and 4 contain plasmids isolated from *E. coli* strains. The plasmids are: pBR322 (4.3kb) in lane 2, pR307 (21.4kb) in lane 3, and pR177 (42.8kb) in lane 4. Lanes 5 contains plasmid isolated from *S. phaeochromogenes*, lane 6 had *S. clavuligerus* DNA, and lane 7 contains DNA isolated from *S. venezuelae* 13s.

of the large *S. coelicolor* plasmid, SCP1 (approximately 223kb). Although there were no indications as to the size of plasmid, if any, in *S. clavuligerus*, a large plasmid might well have been destroyed by the alkaline-SDS procedure. In contrast, the neutral lysis procedure should be suitable for isolation of both large and small plasmids.

Preparations of DNA isolated from cultures, using the Westpheling procedure, were electrophoresed on a 0.7% agarose gel in TEA buffer (Plate 2). Again, λ DNA was used as an indication of the approximate location of chromosomal DNA. It was possible to isolate the plasmids from the *E. coli* strains, seen as bands near the bottom of the gel in lane 2 and above the chromosomal DNA in lanes 3 and 4, but in most cases the preparation were heavily contaminated with chromosomal DNA due to the less selective nature of the neutral lysis procedure. The chromosomal DNA isolated from *S. venezuelae* 13s (lane 5) interfered with the visualization of plasmid DNA and repeated attempts to demonstrate plasmid DNA in *S. venezuelae* 13s by this method were unsuccessful. The neutral lysis procedure was effective in isolating plasmid DNA from *S. phaeochromogenes* (lane 6). With careful examination, a faint plasmid band can be detected in the *S. clavuligerus* preparation (lane 7). The location of this faint band is marked with an arrow.

ii) Modified Neutral Lysis

Since plasmid DNA from *S. clavuligerus* was present in very small amounts, if at all, it seemed appropriate to modify the Westpheling Neutral Lysis procedure to minimize any degradation of DNA that might be occuring. Modifications used were all designed to hasten the procedure and therefore allow less time in which degradation could occur. The neutral lysis procedure was modified in three ways. First, a higher concentration of lysozyme was used in the lysis step in order to hasten lysis of the cells. A shorter incubation time for salt precipitation of chromosomal DNA was also used, as it

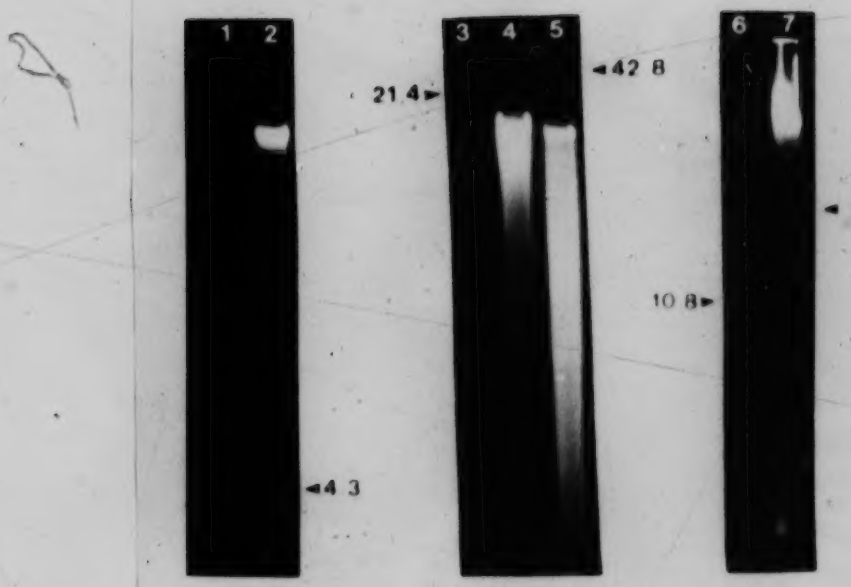


Plate 2: Agarose gel electrophoresis of plasmid DNA samples prepared by the Westpheling neutral lysis. Plasmid DNA preparations were analyzed by electrophoresis on a 0.7% agarose gel in TEA buffer. Lane 1 contains λ DNA, lanes 2, 3, and 4 contain DNA isolated from *E. coli* strains. The plasmids are: pBR322 (4.3kb) in lane 2, pR307 (21.4kb) in lane 3, and pR117 (42.8kb) in lane 4. Lane 5 contains DNA isolated from *S. venezuelae* 13s, lane 6 contains DNA isolated from *S. phaeochromogenes*, and lane 7 contains DNA isolated from *S. clavuligerus*.

appeared that maximal precipitation had already occurred after 10 min., and so the 16h precipitation time was excessive. Also, in conjunction with the shortened time of precipitation, a higher speed was used for centrifugation in order to obtain better separation of the chromosomal DNA from the plasmid DNA. Finally, phenol extractions were used rather than polyethylene glycol precipitation of the DNA. Again, this decreased the amount of time required to complete the preparation, as well as resulting in a cleaner preparation. The shorter length of time used would mean that there was less exposure of the DNA, during the isolation procedure, to any enzymes that could degrade the DNA. Again, the DNA isolated with the procedure was electrophoresed on a 0.7% agarose gel (Plate 3). Lane 1 contains the DNA isolated from *S. clavuligerus*. An extrachromosomal DNA band, as indicated with the arrow (a) is clearly visible. The arrows marked with a (c) indicate the chromsomal DNA for both *S. clavuligerus* (lane 1) and *S. phaeochromogenes* (lane 2). More than one extrachromsomal DNA band is evident in the *S. phaeochromogenes* preparation which may be open circular and linear forms of the plasmid band that was observed clearly in the two previous plasmid preparations (Plate 1 and 2), here indicated with an arrow (b), or there may be more than one plasmid present. The modifications used do not hinder the isolation of plasmid DNA, and in fact with *S. phaeochromogenes* there was an improved yield of plasmid DNA over that seen with the Westpheling Neutral Lysis procedure. Using this procedure it was possible to isolate plasmid DNA from *S. clavuligerus* on an infrequent basis, but no further modifications were found which could enable the "plasmid" to be isolated on a routine basis or with better yields.

IV.2.2.3. Discussion

There was no reproducible evidence of covalently closed circular plasmids, in *S. clavuligerus*, by any of the procedures used. Occassionally, a faint plasmid band was

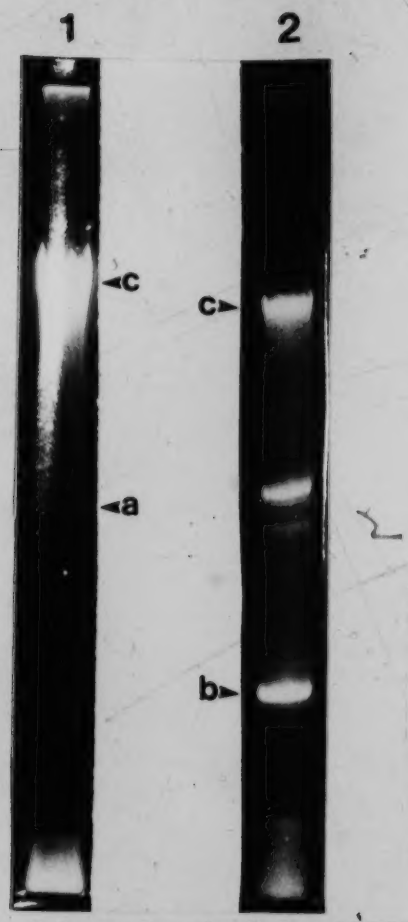


Plate 3: Agarose gel electrophoresis of plasmid DNA samples prepared by the modified neutral lysis procedure. Plasmid DNA preparations were analyzed by electrophoresis on a 0.5% agarose gel in TEA buffer. Lane 1 contains *S. clavuligerus* DNA. Lanes 2 contains *S. phaeochromogenes* DNA. Arrows marked a and b indicate extrachromosomal DNA bands; c indicates chromosomal DNA.

observed on agarose gels of the neutral lysis-type preparations. It was never possible to isolate this "plasmid" on a large scale, and so there is no way of investigating it in more detail. There are several possible explanations; the "plasmid" may be of low copy number and therefore be difficult to isolate. Alternatively, the "plasmid" may be capable of integration into the chromosome, and only rarely exist as an autonomous replicon. There is evidence for this type of phenomenon in other *Streptomyces* spp. (Bibb *et al.* 1981; Cohen *et al.* 1985; Hopwood *et al.* 1984). The existence of such a segment of DNA as a plasmid is considered to be unlikely due to possible incompatibility effects.

IV.3. DEVELOPMENT OF CLONING VECTORS FOR USE WITH *STREPTOMYCES CLAVULIGERUS*

One of the reasons for interest in plasmids of *S. clavuligerus* was for development of cloning vectors, and so a second aim of this research project was to obtain a cloning vector useful in *S. clavuligerus*. Since it appeared that there was not an easily isolatable natural plasmid which could be adapted for use as a cloning vector within *S. clavuligerus*, attention was shifted to the adaptation of broad host range cloning vectors from other *Streptomyces* spp. The plasmid pIJ702 was selected for further study because of its broad host range and reported ability to transform *S. clavuligerus* (Katz *et al.* 1983).

IV.3.1. Transformation of *S. clavuligerus* with pIJ702 from *S. lividans*

Plasmid DNA, from *S. lividans* 3131 bearing pIJ702 (Plate 4), was isolated using a neutral lysis plasmid isolation procedure. The plasmid DNA migrated to a location indicative of its reported size of 5.8kb (Hopwood *et al.* 1985). A large scale neutral lysis plasmid isolation procedure, as described in Materials and Methods, was used to obtain a sufficient amount of plasmid DNA to be used for transformations. Some chromosomal DNA was present, but this was not expected to interfere with

Plate 4: Agarose gel electrophoresis of pIJ702 (5.8kb) prepared by the modified neutral lysis procedure. The plasmid DNA preparation was analyzed by electrophoresis on a 0.7% agarose gel in TEA buffer. The covalently closed and circular (ccc) plasmid DNA is indicated by an arrow.

transformations. The transformation procedure involved the incubation of protoplasts prepared from *S. clavuligerus*, with the isolated plasmid DNA, in the presence of 25% polyethylene glycol 1000. The expected transformation frequency for pIJ702 was 10^5 - 10^6 transformants per μg of DNA, based on literature reports (Katz *et al.* 1983). In transformations using *S. clavuligerus* protoplasts, however, no transformants were obtained (Table 4). There are a number of possible explanations for this failure to observe transformation. Either the plasmid DNA was defective and not able to transform, or the transformation procedure that was being used was unsuitable, or the *S. clavuligerus* culture was incapable of being transformed. As a check of both the quality of the plasmid DNA and the appropriateness of the procedure, the same transformation procedure was carried out using protoplasts of *S. lividans* 1326, a plasmid free strain instead of *S. clavuligerus*. The observed frequency of transformation of 8.0×10^5 transformants per μg DNA indicated that both the quality of the plasmid DNA and the transformation procedure itself were able to support high levels of transformation in *Streptomyces* spp. This suggested that the problem in transformations using *S. clavuligerus* protoplasts was due to an inability of the *S. clavuligerus* protoplasts to be transformed by the *S. lividans* derived plasmid. Previous studies by Bailey *et al.* (1984) using pIJ702 as a cloning vector, stated that plasmid DNA from *S. clavuligerus* was used for the transformation procedures. This specification as to the source of the plasmid DNA suggested that *S. clavuligerus* might have a restriction system that was preventing the maintenence of pIJ702 derived from *S. lividans*.

IV.3.2. Isolation of pIJ702 from *S. clavuligerus*

In order to isolate pIJ702 plasmid DNA from *S. clavuligerus*, it was necessary to first introduce the plasmid into *S. clavuligerus* and have it be maintained. Assuming that a restriction/modification enzyme system was responsible for the inability to achieve

Table 4: Transformation of *S. clavuligerus* and *S. lividans* with pIJ702 isolated from *S. lividans*

Vector	Transformants/μg DNA	
	S. clavuligerus	*S. lividans*
pIJ702 from *S. lividans* 3131	0	8.0×10^5

transformants, a method was required to overcome the barrier to the heterologous DNA. The *S. clavuligerus* cells had to be treated in a manner so as to inactivate the restriction enzyme, while not altering or inactivating the modification enzyme. Two ways of achieving this goal were investigated: selective inactivation of restriction enzyme(s) by mild heat treatment and generation of mutants defective in restriction enzyme(s).

IV.3.2.1. Transformation of Protoplasts after Heat Shock Treatment

Protoplasts of *S. clavuligerus* were exposed to 45°C for 5 min to 10 min just prior to transformation as suggested by Dr. I. Hunter (personal communication). The brief incubation at 45°C should heat inactivate the restriction enzyme(s) without affecting the modification system so that the plasmid can enter the cell, be modified, and maintained. Using 0.3μg of *S. lividans* derived pIJ702 to transform *S. clavuligerus* protoplasts that had been heat treated, 20 transformants were obtained. Plasmid DNA was isolated from the 20 *S. clavuligerus* transformants by the procedure of Kieser *et al.* (1982). All contained a plasmid of the same mobility as pIJ702.

IV.3.2.2. Transformation of Protoplasts Derived from Mutagenized Spores

Spores of *S. clavuligerus* that had been treated with NTG were inoculated into phytone seed medium, and then transferred to phytone seed medium containing glycine, to make protoplasts as described in Materials and Methods. The protoplasts were transformed with *S. lividans* derived pIJ702 plasmid DNA using the same transformation procedure as before. Mutagenized cells were used to prepare protoplasts in hopes that the random mutagenesis procedure might have produced some mutants deficient in restriction enzyme(s). When 0.3 μg of pIJ702 plasmid DNA isolated from *S. lividans* was used to transform the protoplasts, three transformants were obtained. When examined for plasmid content, two of the transformants contained plasmid DNA of the same mobility as pIJ702, while the third contained a plasmid with lower mobility.

Plate 5: Agarose gel electrophoresis of pIJ702 and the lower mobility plasmid isolated from mutagenized cultures of *S. clavuligerus* after transformation with pIJ702. The DNA preparations were analyzed by gel electrophoresis on a 0.7% agarose gel, in TEA buffer. Lane 9 contains λ DNA, and lane 8 contains DNA isolated from *S. clavuligerus* by the modified neutral lysis method (prepared November 1982), showing the faint plasmid band, marked with an arrow. Lanes 1 and 7 contain the original pIJ702 as isolated from *S. lividans*. Lanes 2 through 5 contain the lower mobility plasmid, with twice the amount of the DNA preparation applied in lanes 4 and 5.

This lower mobility plasmid was analyzed by gel electrophoresis on a 0.7% agarose gel, in TEA buffer, along with pIJ702 and λ DNA (Plate 5). The λ DNA (lane 9) indicates the approximate location of chromosomal DNA for the other DNA samples. The pIJ702 plasmid, as isolated from *S. lividans* is seen near the bottom of the gel (lanes 1 and 7). The plasmid isolated from the transformed mutagenized *S. clavuligerus* isolate, called pIJ702-SC, has a lower mobility (lanes 2, 3, 4, and 5). The amount of material applied to the gel was 20 μL of the preparation (lanes 2 and 3), and 40 μL (lanes 4 and 5). A sample of one of the original plasmid isolation preparations of *S. clavuligerus* which showed a faint plasmid band was also placed on the gel to ensure that the plasmid band observed earlier (Plate 3) was not pIJ702-SC.

i) Restriction Endonuclease Digests of pIJ702-SC

To determine the size difference between pIJ702 and pIJ702-SC, single digests were performed using four different restriction endonucleases, *Bcl* I, *Sal* I, *Sma* I, and *Sst* II. The pIJ702-SC was isolated using a large scale neutral lysis procedure, and CsCl-ethidium bromide density gradient centrifugation, as described in Materials and Methods. The digests were then performed on both pIJ702 and pIJ702-SC, using the digestion conditions as stated earlier. Samples from the digestions were electrophoresed on a 1.25% agarose gel with the addition of a λ–*Bst* EII digest as markers, and stained with ethidium bromide (Plate 6). Digestions with *Sst* II (lanes 2 and 3) showed two different fragments in pIJ702-SC as compared to pIJ702. Digestion with *Sma* I (lanes 4 and 5) also gave rise to two new fragments. The *Sal* I digests (lanes 6 and 7) generated three new fragments, and the *Bcl* I digests (lanes 8 and 9) resulted in two new fragments, and twice as intense staining of an already existing fragment. The double intensity indicates that the concentration of the fragment has doubled. The two

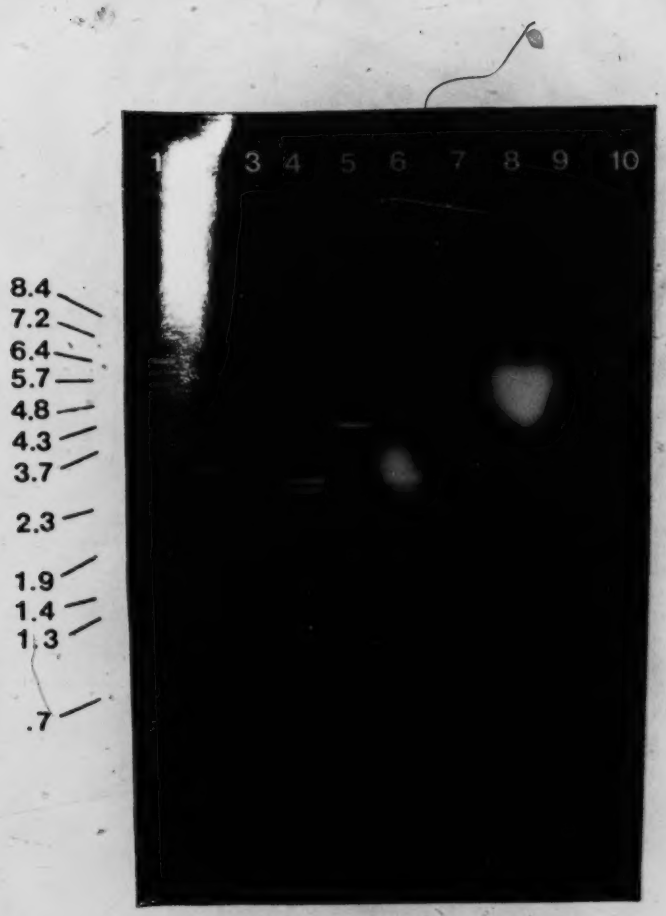


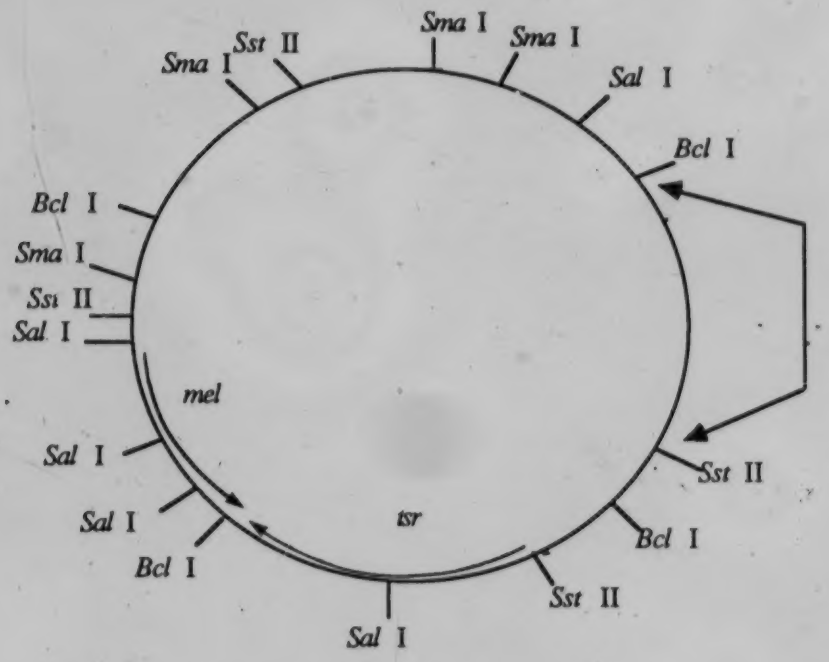
Plate 6: Analysis of restriction enzyme digests of pIJ702-SC and pIJ702 by agarose gel electrophoresis. The digests were electrophoresed on a 1.25% agarose gel in TEA buffer. Lanes 1 and 10 contain a λ-*Bst* EII digest as size markers with the fragment sizes indicated in kb. Lanes 2, 4, 6 and 8 are digests of pIJ702-SC with: lane 2, *Sst* II; lane 4,*Sma* I; lane 6, *Sal* I; and lane 8, *Bcl* I. Lanes 3, 5, 7 and 9 are digests of the original pIJ702 with: lane 3, *Sst* II; lane 5, *Sma* I; lane 7, *Sal* I; and lane 9, *Bcl* I.

Table 5: Sizes of fragments obtained after restriction digests of pIJ702 and pIJ702-SC.

Restriction enzyme used in digest	Sizes of fragments (in base pairs) pIJ702 (literature value)[a]	pIJ702 (apparent)	pIJ702-SC (apparent)
Bcl I	1770	1770	1770
	1560	1560	1560
	1425	1340	1430
	1085	1100	2 x 1100
			390
Total	5840	5770	7350
Sal I	2380	2380	2060
	2130	2060	1750
	550	610	1070
	515	510	970
	265	260	610
			510
			260
Total	5840	5840	7230
Sma I	4280	4200	2750
	840	910	2580
	500	430	910
	220	220	430
			220
Total	5840	5760	6890
Sst II	2460	2380	3000
	1735	1750	1750
	1210	1210	1210
	435	480	750
			480
Total	5840	5820	7190

a. Hopwood *et al.* (1985)

Figure 2: Restriction Enzyme Sites of pIJ702, and the Point of Insertion of the Extra Fragment of DNA.



Both pIJ702 and pIJ702-SC were digested with the same enzymes, and the resulting fragments were compared to those from the literature (Hopwood *et al.* 1985). The point of insertion of the "new" segment of DNA was determined by examination of the fragments from the digestion of pIJ702-SC which were altered as compared to pIJ702, and is between the *Bcl* I and *Sst* II restriction endonuclease sites as indicated

flares on the gel are areas at which ethidium bromide was concentrated and did not rinse off; they do not indicate areas of DNA. All of the digests appeared to be complete except for the *Sal* I digest of pIJ702-SC (lane 6) which showed a faint ladder of high molecular wieght fragments due to incomplete digestion.

For each restriction endonuclease that was tested, the fragments that were obtained from pIJ702-SC were compared to those obtained from pIJ702. The size of each frogment was determined using the λ-*Bst* EII digest size markers and then compared to the known sizes of the fragments of pIJ702, as determined by Hopwood *et al.* (1985) (Table 5). The results for pIJ702 correlated well with those obtained by Hopwood, while pIJ702-SC was determined to be approximately 1.4kb larger than pIJ702. Using the known map for pIJ702 from Hopwood *et al.* (1985), it was possible to determine an approximate location for the insertion of the new DNA fragment (Figure 2). The area within which the new fragment of DNA had become inserted is between the *Bcl* I site and the *Sst* II site as indicated by the arrows.

ii) Hybridization Studies

The extra segment of DNA in pIJ702-SC was postulated to have two possible origins: the fragment of DNA could have resulted from a duplication of a fragment of the original pIJ702 plasmid; the fragment could have come from the DNA of *S. clavuligerus*. This latter possibility is complicated by the recent observation of Keen (1985) that *S. clavuligerus* contains extrachromsomal DNA. When total DNA was prepared from *S. clavuligerus* by the method of Fishman and Hershberger (1983) it was possible to separate plasmid DNA from chromosomal DNA on an agarose gel. This DNA isolation method is based on a neutral lysis procedure, but with the addition of a protein digestion step, using proteinase K, prior to the salt precipitation. The plasmid detected by this method is apparently linear and contains protein which is

Plate 7: Agarose gel electrophoresis of linear plasmid from *S. clavuligerus*. The DNA preparations were analyzed by electrophoresis on a 0.7% agarose in TEA buffer. Lane 1 contains DNA isolated from *S. clavuligerus* single spore isolate #10, and lane 2 contains DNA isolated from *S. clavuligerus* NRRL3585. The plasmid DNA is marked with an arrow.

Plate 8: Agarose gel electrophoresis of the fractions from the sucrose gradient used to isolate linear plasmid from *S. clavuligerus*. The fractions were electrophoresed on a horizontal 0.7% agarose gel in TEA buffer. The lane numbers are indicated on the plate. Every second fraction collected was electrophoresed, with the first fraction, lane 1, from the top of the sucrose gradient. Plasmid DNA was determined to be contained in fractions 11 through 19 at the highest concentrations, lanes 6 through 10.

removed by the proteinase K digestion. The combination of these two unusual features of the plasmid resulted in an inability to isolate the plasmid by commonly used plasmid isolation procedures such as were used earlier in this study. However, in investigating the possibility that the extra DNA fragment in pIJ702-SC had come from *S. clavuligerus* DNA, it seemed advisable to distinguish between chromosomal and linear plasmid DNA.

The procedure used by Keen (1985) as described in Materials and Methods for the isolation of linear plasmid DNA was followed, and the DNA preparation was electrophoresed on a 0.7% agarose gel (Plate 7). Lane 1 contains DNA isolated from the single spore isolate #10, and lane 2 contains DNA isolated from the original parent culture, *S. clavuligerus* NRRL 3585. As can be seen, both contain the linear plasmid (marked with an arrow). The plasmid was further purified by sucrose gradient centrifugation. Samples of every second fraction from the sucrose gradient were analyzed on a 0.7% agarose gel to locate the linear plasmid material (Plate 8). Fractions 11 through 19 inclusive were found to be greatly enriched for linear plasmid DNA although a background smear of chromosomal DNA fragments was also present. These fractions were saved for restriction endonuclease digests and hybridization studies.

In order to investigate the possibility that the new DNA fragment in pIJ702-SC had arisen from the linear plasmid of *S. clavuligerus*, digests of pIJ702 and pIJ702-SC, prepared exactly as in Plate 6, were transferred from the agarose gel to nitrocellulose and then hybridized with labelled linear plasmid DNA. The linear plasmid was labelled with ^{32}P by nick-translation, and used to probe the nitrocellulose, as described in Materials and Methods. The nitrocellulose was then exposed to x-ray film in order to detect fragments from the digests which hybridized to the probe (Plate 9). Lanes 3, 5, 7, and 9 contained digests of pIJ702-SC. The fragments in these lanes hybridized to the linear plasmid probe, whereas the digests of pIJ702 showed no hybridization. Furthermore,

Plate 9: Autoradiogram of the Southern transfer of the DNA from the gel (Plate 6) after hybridization with ^{32}P-labelled-linear plasmid. This is the reverse of the gel pictured on Plate 6 with lane 1 here, corresponding to lane 10. Lanes 2, 4, 6, and 8 contain the digests of pIJ702, while lanes 3, 5, 7, and 9 contain the digests of pIJ702-SC. The lanes which hybridized to the probe are lane 3, *Bcl* I digest of pIJ702-SC; lane 5, *Sal* I digest of pIJ702-SC; lane 7, *Sma* I digest of pIJ702-SC; and lane 9, *Sst* II digest of pIJ702-SC.

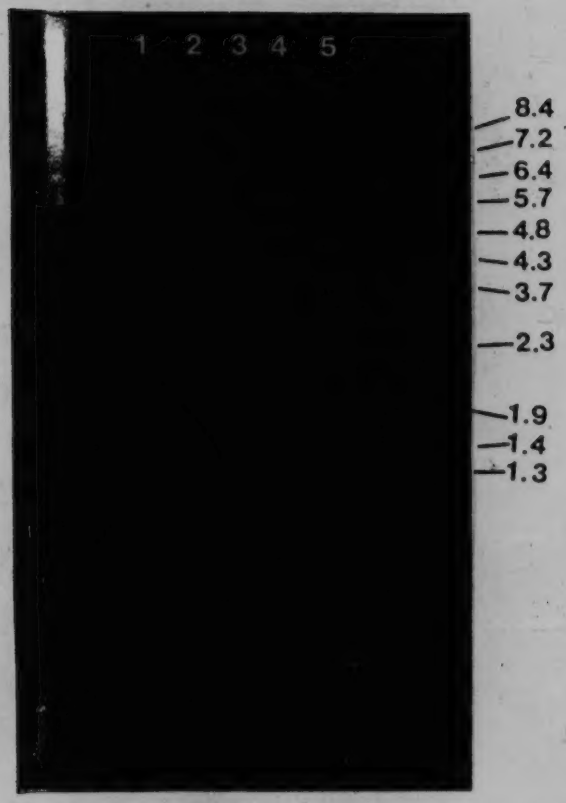


Plate 10: Agarose gel electrophoresis of restriction enzyme digests of *S. clavuligerus* linear plasmid DNA. Samples of DNA were electrophoresed on a 1.25% agarose gel in TEA buffer. Lane 1 contains a *Bcl* I digest; lane 2 is *Sal* I; lane 3 is *Sma* I; and lane 4 is *Sst* II. Lane 5 is a *Bst* E II digest of λ as a marker lane with the fragment sizes indicated in kb.

the restriction fragments of pIJ702-SC which showed hybridization corresponded to the fragments which were different from those of pIJ702. These data suggested that the fragment that increased the size of pIJ702 originated from the linear plasmid of *S. clavuligerus*. Looking back at Plate 8, however, it was observed that fragments of chromosomal DNA were present in the linear plasmid preparation. This chromosomal DNA was labelled together with the linear plasmid, and so the observed hybridization to the fragments of pIJ702-SC could also be due to hybridization with the small amount of contaminating chromosomal DNA.

In order to determine whether chromosomal DNA or linear plasmid DNA was responsible for the hybridization, a reciprocal type of hybridization was performed. In this case, restriction endonuclease digests of *S. clavuligerus* linear plasmid and chromosomal DNA were subjected to agarose gel electrophoresis, transferred to nitrocellulose and then hybridized with labelled pIJ702-SC. The digests of the linear plasmid were first analyzed on a separate gel, not used for the hybridization, in order to observe the digest patterns (Plate 10). A sample of λ DNA digested with *Bst* E II was included on the gel as a marker (lane 5). The restriction enzymes used to prepare digests of the linear plasmid were with *Bcl* I (lane 1), *Sal* I (lane 2), *Sma* I (lane 3), and *Sst* II (lane 4). Since the linear plasmid is small relative to the chromosomal DNA, it gives rise to relatively few fragments, and there are more copies of each fragment of the plasmid DNA per μg DNA than of the chromosomal DNA. In order to obtain similar amounts of all fragments on the gel to be used for the hybridization, it was necessary to apply 1000x less plasmid DNA than chromosomal DNA; for this reason, it was difficult to observe the digests of the linear plasmid on the stained gel that was used for the hybridization (Plate 11a). The fragments from the digests of the linear plasmid are barely detectable (lanes 2 through 5). The chromosomal DNA digests of *S. clavuligerus* can be clearly

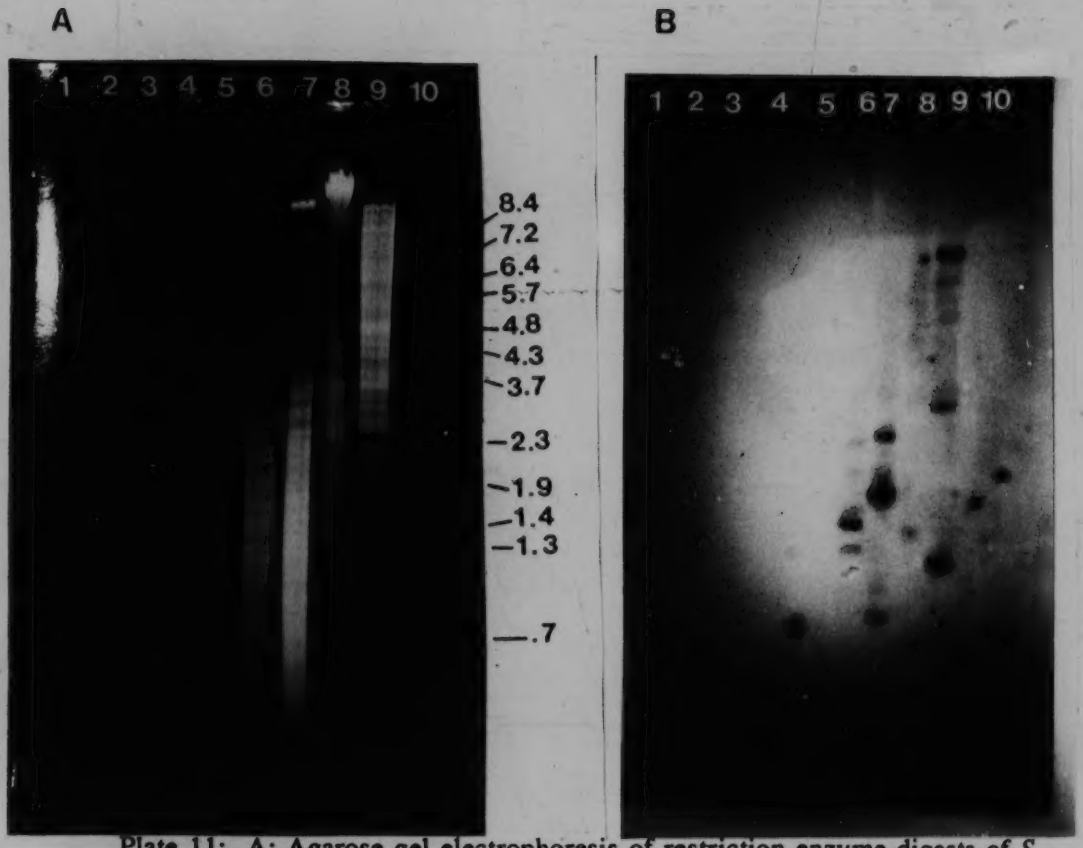


Plate 11: A: Agarose gel electrophoresis of restriction enzyme digests of *S. clavuligerus* linear plasmid DNA and chromosomal DNA. The digests werre analyzed by electrophoresis on a 1.25% agarose in TEA. Lanes 2 through 5 contain digests of linear plasmid DNA, lanes 6 through 9 contain digests of chromosomal DNA. Lanes 2 and 6 are *Bcl* I digests; lanes 3 and 7 are *Sal* I; lanes 4 and 8 are *Sma* I; and lanes 5 and 9 are *Sst* II. Lane 10 is a *Bst* E II digest of λ DNA used as a marker.

B: Autoradiogram of the Southern transfer of the DNA from the gel after hybridization with ^{32}P-labelled-pIJ702-SC. This photographic plate is in the same orientation as the gel in A. Lanes 2 through 5 are the corresponding digests of the linear plasmid. Lanes 6 through 9 are digests of chromosomal DNA with lane 6, *Bcl* I; lane 7, *Sal* I; lane 8, *Sma* I; and lane 9, *Sst* II.

observed (lanes 6 through 9). The *Bst* E II digest of λ DNA was used as a marker lane (lane 10). The gel with all of the digests (Plate 11A) was transferred to nitrocellulose as described in Materials and Methods. The plasmid pIJ702-SC was labelled with ^{32}P by nick translation, and then used to probe the nitrocellulose paper. The paper was then exposed to X-ray film to locate hybridizing bands (Plate 11B).

With the second hybridization, it was observed that the ^{32}P-labelled-pIJ702-SC was only able to hybridize to the fragments of DNA in the chromosomal digests of *S. clavuligerus*. This indicates that the extra sequence of DNA in pIJ702-SC actually originated from some area on the chromosome of *S. clavuligerus*. The hybridization previously observed to the linear plasmid DNA preparation must therefore have resulted from the presence of contaminating chromosomal DNA.

IV.3.3. Transformation of *S. clavuligerus* with Modified pIJ702

As a result of the heat shock and mutagenesis procedures, described earlier, pIJ702 was successfully introduced into *S. clavuligerus* and pIJ702 and pIJ702-SC were reisolated from *S. clavuligerus*. If assumptions of the presence of a restriction/modification system are correct, the plasmids reisolated from *S. clavuligerus* should be much more efficient in transforming *S. clavuligerus* protoplasts than was plasmid derived from *S. lividans*. The plasmids tested for transformation efficiency in *S. clavuligerus* were the plasmid DNA obtained from one of the transformants after heat-shock treatment (apparently normal pIJ702), and pIJ702-SC. The results of the transformation of *S. clavuligerus* protoplasts, using the same transformation procedure as was used previously, indicated that the two modified plasmids were capable of transforming protoplasts of *S. clavuligerus* with high frequency (Table 6). The plasmid pIJ702-SC, obtained from a single NTG-treated transformant gave rise to 5.4×10^6

transformants/μg DNA. The pIJ702 isolated from the heat-shock treated transformant produced 1.4 x 10^6 transformants/μg DNA. Both of these transformation frequencies exceeded 10^5/μg DNA, which is the transformation rate expected for transformation of *S. clavuligerus* with pIJ702 from *S. clavuligerus* (I. Hunter, personal communication).

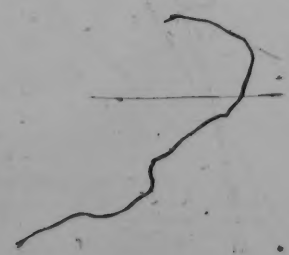
It should be noted that pIJ702-SC could also retransform *S. lividans*, and maintain its larger size. Also, both the thiostrepton resistance gene and the tyrosinase gene were expressed in *S. lividans*.

IV.3.4. Transformation of Cured *S. clavuligerus* with pIJ702 from *S. lividans*

The transformant from which pIJ702-SC was isolated, here named NTG-13, was cured of the plasmid by growth on nonselective media. Spores of NTG-13 were grown in phytone seed medium, in the absence of thiostrepton, and then plated onto phytone seed medium agar plates. Colonies were then replica plated to plates of phytone seed medium, in the presence and absence of thiostrepton to determine which colonies had been cured of the plasmid. Of 64 colonies tested, all were thiostrepton sensitive, indicating that the plasmid had been lost. Once the plasmid had been lost, it was possible to retransform the protoplasts with pIJ702 isolated from *S. lividans*. The transformation frequency observed when protoplasts from this so called restriction deficient isolate of *S. clavuligerus* were challenged with heterologous DNA, was 10^3 transformants per μg of pIJ702. The number of transformants obtained per μg of DNA is lower than if there was no restriction barrier, and yet greater than that obtained before treatment to generate possible restriction deficient protoplasts. The results indicate that NTG-13 is partially restriction deficient.

Table 6: Transformation of *S. clavuligerus* with pIJ702 and pIJ702-SC

Plasmid	Transformants/μg DNA
pIJ702-SC	5.4×10^6
pIJ702 from a heat-shock treated transformant	1.4×10^6



IV.3.5. Discussion

It was found that *S. clavuligerus* has a restriction system that prevents the transformation of protoplasts with heterologous DNA. The restriction system can be overcome to a certain extent either by treating the protoplasts with heat just prior to transformation to inactivate the restriction enzyme, or by mutagenizing spores of *S. clavuligerus* prior to making protoplasts to obtain restriction deficient protoplasts. The restriction deficient protoplasts were not completely devoid of restriction activity, as the frequency of transformation obtained was not as high as would be expected with homologous DNA where restriction is not involved (10^5 - 10^6 transformants/μg DNA). Instead it was 10^3 transformants per μg DNA, indicating that they were partially restriction deficient.

The linear plasmid, at this time, has not been shown to have any involvement with antibiotic production. It is a possibility worth exploring, however, as there is evidence of the involvement of a linear plasmid in the production of lankacidin antibiotics (Hayakawa *et al.* 1979). Also, the unusual properties of the linear plasmid explain why this study and previous surveys of *Streptomyces* spp. (Kirby *et al.* 1982) were unable to detect the presence of plasmid DNA in *S. clavuligerus*.

Plasmid DNA reisolated from *S. clavuligerus* after heat shock or NTG mutagenesis induced transformation with pIJ702 from *S. lividans*, normally was indistinguishable from the original pIJ702 by agarose gel electrophoresis. The pIJ702-SC obtained after transformation of one particular restriction deficient protoplast was about 1.4kb larger that the pIJ702 that was used for the transformtion. The segment of DNA that inserted itself into the plasmid apparently came from the chromosome of *S. clavuligerus*, and could be the result of a transpositional event. If this is true, it would be the first recorded incidence of an insertion sequence in *Streptomyces clavuligerus*.

V. CONCLUSION

The initial goal of this project was to determine if a plasmid existed in *S. clavuligerus* NRRL 3585, if it would be suitable for use as a cloning vector, and if it was involved in some manner in the production of beta-lactam antibiotics. The basis for this interest was the discovery of the *S. coelicolor* A3(2) plasmid, SCP1, and demonstration of its direct involvement in the production of methylenomycin A (Hornemann and Hopwood 1981). Criteria useful for determining plasmid involvement were outlined by Hopwood (1978). The first criterion tested was the loss of the ability of isolates to produce antibiotic, or an alteration of the levels of antibiotic produced, through treatment with curing agents. Isolates were obtained through protoplast formation and regeneration or novobiocin treatments, which appeared to have altered levels of production ~~of a~~ntibiotic or clavulanic acid, and in some instances these changes were correlated with a lack of aerial mycelia, giving "bald" colonies. However, these changes were transient and seemed more likely to be related to the stresses of the curing treatment. In no instance was a stable non-producing isolate obtained after a curing technique, which suggests that plasmids are not involved in the production of antibiotics in *S. clavuligerus*.

Results from curing techniques alone are insufficient evidence to discount the involvement of plasmids, so attempts were made to demonstrate physically the presence of plasmids. Two isolation procedures were used, one involving lysis with SDS under alkaline conditions and the other involving a neutral lysis procedure. The results indicated that a covalently closed circular plasmid is not present in *S. clavuligerus* or at least not isolated by these methods, and therefore would not be involved in the production of antibiotics. However, with the addition of a preliminary protein digestion step to a procedure for total DNA isolation (Keen 1985), it is possible to isolate what appears to be a linear plasmid. Confirmation of the presence of a linear plasmid in *S. clavuligerus* has been obtained from Y. Aharonowitz's laboratory at the University of Tel Aviv (Aharonowitz, personal communication). The linear plasmid is 12.8 kb in

size. The function and copy number per genome of this plasmid is not known. The appearance of linear plasmids in *Streptomyces* spp. cannot be considered an isolated incident (Hayakawa *et al.* 1979). It would be interesting to determine the distribution of linear plasmids in *Streptomyces* spp., and their characteristics, including transmissibility, and mode of replication. More importantly, it would be interesting to determine their function.

A second aim of this study was to establish an efficient transformation system within *S. clavuligerus* in order to facilitate gene cloning. Poor efficiency of transformation was obtained using the plasmid pIJ702 isolated directly from *S. lividans* to transform *S. clavuligerus*. It was clear that the plasmid was not maintained in *S. clavuligerus*, leading to the assumption that the plasmid DNA was being digested.

Two methods were used to overcome the problem of restriction. The NTG mutagenesis gave rise to three isolates which were successfully transformed with foreign DNA due to deficiencies in their restriction enzyme systems. Since transformation of these isolates with pIJ702 isolated from *S. lividans* did not take place at optimal frequencies, 10^5 - 10^6 transformants per µg DNA (Hopwood *et al.* 1985), it is likely that more than one restriction system is present in the wild-type strain and that these isolates are only partially defective in restriction ability. Plasmid DNA obtained from transformants of these isolates was used to transform wild-type *S. clavuligerus* and optimal frequencies were observed, indicating that the plasmid had been modified and was no longer subject to restriction by *S. clavuligerus*.

The procedure of heat shocking protoplasts (Hunter, personal communication) also gave rise to transformants which failed to restrict the foreign pIJ702. The plasmid subsequently isolated from these transformants was also able to transform wild-type *S. clavuligerus* at optimal frequencies. So, through the use of NTG mutagenesis or heat shock treatment it has been possible to transform *S. clavuligerus* with foreign plasmid DNA. Plasmid DNA subsequently reisolated from these restriction-deficient or heat

shock treated strains of *S. clavuligerus* was able to transform wild-type cultures at optimal frequencies. These same procedures could be used with any number of potentially useful cloning vectors.

As a result of the transformation of one of the restriction deficient isolates of *S. clavuligerus* with pIJ702 isolated from *S. lividans*, a plasmid was isolated from *S. clavuligerus* that had increased in size by 1.4 kb over the original pIJ702. Upon examination it was determined that a segment of DNA from the *S. clavuligerus* chromosome had become inserted into the original pIJ702. The event that took place resembles a transposition event, and so may represent evidence for transposable elements in *Streptomyces* spp. The use of transposable elements in cloning procedures and mutagenesis has been amply demonstrated in other species and could be expected to alter significantly the nature of genetic research in *Streptomyces* spp.

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